REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

I. CLAIM STATUS AND AMENDMENTS

Upon filing the present Request for Continued Examination (RCE), Claims 1, 4, 5, 8- 10, 13-17, 20, 21, and 24-27 stand withdrawn from consideration; Claims 2, 3, 6, 7, 11, 18, 19, 22, and 23 were cancelled without prejudice or disclaimer and Claim 12 remains pending. In this amendment, Applicants have cancelled Claim 12 and present Claims 28-52 for examination on the merits.

Support for new Claims 28-52 can be found throughout the specification and the claims as originally filed in the application. For example, the abstract states that the provided compositions are useful as drugs to promote granulation formation, especially as a preventative and curative agent for skin ulcers. [0026] states that a human recombinant HGF wherein five amino acid residues are deleted in the first Kringle domain (dHGF / SEQ. ID. No. 1) is excellent in promoting granulation formation by exogenous supplementation and is particularly excellent in treating a skin ulcer resulting from diabetes. [0107] states that the present drug of the present invention contains the dHGF as the active ingredient and [0108] states that the drug of the present invention can be formulated into several forms including, but not limited to, ointments, creams, gels, lotions, and suspensions. More specifically, [0113] states that the drug of the present invention can be in the form of an ointment; [0114] and [0116] states that the drug of the present invention can be in the form of a gel, which may include a gelling agent; [0115] states that the drug of the present invention can be in the form of a cream, which may include an antiseptic and fatty acid ester; and [0117] states that the drug of the present invention may be in the form of a liquid, which may include an antiseptic, gelling agent, and fatty acid ester. [0122] states that the when the granulation-formation-promoting agent of the present invention is used as an external preparation it may be used with a variety of wound covering agents and [0123] states that the external preparation may be contained within the wound covering material or added after the wound covering material is applied to the damaged site.

The specification also contains working examples that support the present claims. For instance, Examples 2, 4, and 5 demonstrate the selection of mammals having a skin ulcer for receiving a drug that promotes granulation formation, neovascularization, and enhanced wound healing, topically administering a drug that comprises dHGF to the skin ulcers, and subsequently analyzing granulation formation, neovascularization, and enhanced wound healing at the skin ulcer. [0129] states that after the ulcer was created on the experimental animal, a wound covering material was applied and that the dHGF was administered to the ulcer thereafter. [0135] states that it was recognized that the application of dHGF enhanced formation of granulation tissue in a dose-dependent manner. [0136] states that it was observed that skin ulcer enclosure rate was enhanced by administration of dHGF. Accordingly, Applicants submit that the present claims are fully supported by the specification and that no new matter has been added by the present amendments.

II. INTERVIEW SUMMARY

Applicants thank the Examiner for the courteous interview held on November 18, 2008 and the helpful comments made therein. Applicants note that a proposed claim set was discussed and Applicants appreciate the Examiner's comments with respect to demonstrating support for all of the claim limitations in the newly presented claims and the need for references to demonstrate the state of the art for HGF biological activities, the differences between dHGF and HGF, and the unpredictability of relying on HGF gene expression data to indicate the biological response to topical administration of HGF protein.

III. CLAIM OBJECTIONS

The Examiner has objected to Claim 12 for containing a grammatical error. In response, Applicants have cancelled Claim 12 without prejudice or disclaimer and now present new Claims 28-52 for examination on the merits.

IV. CLAIM REJECTIONS UNDER 35 USC SEC. 112

The Examiner has rejected Claim 12 for lack of written description and enablement. Specifically, the Examiner states that the Applicants are not in possession of the full scope of Claim 12 and that while being enabling for SEQ. ID. NO. 1, the specification does not

reasonably provide enablement for the full scope of the claim. In an effort to expedite prosecution of the present application, Applicants have cancelled Claim 12 without prejudice or disclaimer and reserve the right to pursue the subject matter of the cancelled claim in continuing applications. Accordingly, Applicants respectfully request that the rejections under 35 USC sec. 112 be withdrawn.

V. CLAIM REJECTIONS UNDER 35 USC SEC. 103

The Examiner has rejected claim 12 under 35 USC sec. 103(a) for being obvious in light of the teachings of Toyada et al. in view of Seki et al.; Nakamura et al.; (US 5,342,831); Nakamura et al., (EP 461,560A1); Yoshida et al. and either Morishita et al (US 7,247,620) or Morishita et al. (WO 02/089854).

According to the Examiner, Toyoda et al. discloses that over expression of HGF in transgenic mice promotes granulation and that the reference describes topical application of HGF to skin wounds to promote granulation formation but that the reference does not disclose dHGF (SEQ. ID. No. 1). The Examiner states that Seki et al., discloses dHGF (SEQ. ID. No. 1) and that this variant of HGF has the same biological properties as HGF (SEQ. ID. No. 3), noting that dHGF binds antibodies to HGF. The Examiner states that Nakamura et al. (US 5,342,831) discloses using HGF to treat skin ulcers and that Nakamura et al. (EP 461,560A1) discloses dHGF (SEQ. ID. No. 1) and that dHGF has the same biological activities as HGF. The Examiner states that Yoshida et al. establish that antibodies directed to HGF inhibit granulation formation and that Morishita et al (US 7,247,620) or Morishita et al. (WO 02/089854) disclose treating diabetic skin ulcers by topical administration of an HGF gene to promote granulation. The Examiner concludes that it would be obvious to substitute dHGF (SEQ. ID. No. 1) as taught by Seki et al or Nakamura et al. (EP 461,560A1) to treat skin ulcers as suggested by Morishita et al., Nakamura et al. (US 5,342,831) and Toyoda et al. and that based on the relationship of HGF and granulation reported by Toyoda et al., Morishita et al., and Yoshida et al. and the teachings of Nakamura et al. (EP 461,560A1) and Seki et al. one of skill in the art would have expected dHGF (SEQ. ID. No. 1) to have this biological activity.

For more than a decade, investigators have sought to develop a topical drug having HGF to treat skin ulcers. The present application discloses that among HGFs, a human recombinant HGF, wherein five amino acid residues are deleted in the first Kringle domain (dHGF) is

excellent in the action of promoting granulation formation and neovascularization by exogenous supplementation and that this dHGF is particularly effective at treating diabetic skin ulcers (see [0026]).

Toyoda et al., published in 2001, developed a transgenic mouse model that over expressed HGF. The authors state that the *in vivo* effects of HGF during skin wound healing are poorly understood (*see last paragraph of Introduction on page 95).* The authors report that in their mouse model, HGF was strongly expressed in the skin of the transgenic mice and that these mice exhibited highly accelerated granulation tissue formation relative to control mice (*see first two paragraphs of Results on page 96).* However, the authors also report that HGF over expression did not overtly affect wound healing and that they saw enhanced fibroblast proliferation (cells that do not express the c-met receptor) in transgenic granulation tissue (*see last two paragraphs on page 99).* The authors hypothesize that the observed fibroblast proliferation was due to highly organized new blood vessels on the supply of nutrients and oxygen to the cells rather than a direct effect of HGF (*Id.).* The authors also note that markedly thicker collagen deposition was noted in their mouse model *(Id.).*

If fibroblast proliferation and increased collagen deposition are secondary responses to the presence of HGF at the skin, as Toyoda et al suggest, one would also expect to see fibroblast proliferation and increased collagen deposition when HGF protein is administered. This is not the case, however. Nakamura US 5,342,831, reports that HGF protein does not have fibroblast growing activity (*see abstract)* and, in contrast to the findings of Toyoda et al., Nakamura US Patent 6,303,126 (**Exhibit A**) discloses that HGF protein administration accelerates collagen decomposition. Furthermore, when taken in context to the rest of the paragraph, Toyoda et al.'s, conclusion that HGF should be applied locally relates to the previously stated concerns regarding the toxic effects of high levels of HGF (*see last paragraph of page 99).* This passage in no way indicates that topical administration of HGF can promote granulation formation and based on the levels of HGF expressed at the surface of the skin and the contradictions above with regard to fibroblast proliferation and collagen deposition, one would not reasonably arrive at the conclusion that Toyoda et al's mouse model is predictive of the biological response when HGF is administered topically.

Applicants also submit that Toyoda et al's finding that even in the presence of large quantities of HGF at the skin surface wound closure was not evident is in contrast to the

teachings of the present application, which shows that topical administration of dHGF promotes wound healing [*see paragraph 0136]*. Importantly, the findings of Toyoda et al. illustrate that over expression of the HGF gene in skin cells results in a drastically different biological response than topical application of the HGF protein. Accordingly, one of skill in the art would not read Toyada et al. as reasonably predicting that topical application of HGF would promote granulation formation and wound healing much less that topical application of dHGF would promote granulation formation and wound healing.

Applicants also respectfully point-out that the disclosures relied on by the Examiner for the proposition that dHGF and HGF have the same biological activities do not represent the state of understanding in the art at the time of filing of the present application. Several references more contemporary than Nakamura et al. (EP 461,560A1) and Seki et al. report that dHGF and HGF have significantly different structures and functions. In fact, references more contemporary than Nakamura et al. (EP 461,560A1) and Seki et al. teach away from topical administration of a drug comprising dHGF protein. Based on transient expression experiments conducted in COS-1 cells, Seki et al. (published in 1990), conclude that the structural difference in dHGF has no effect on biological activity in vitro. (*See 1st paragraph page 326*). Similarly, based on transient expression experiments conducted in hepatocytes, Nakamura et al. (EP 461,560A1, published in 1991) report that recombinant dHGF has noticeable promoting activity on the growth of rat hepatocytes (*see page 5, column 8, lines 43-47*). Shima et al., (published in 1994, **Exhibit B**) report that the deletion in dHGF significantly altered its biological activities, solubility, and immunological properties (*see abstract).* Shima et al. report that HGF is more than 70 fold more soluble than dHGF and that the deletion caused a tertiary structural change that may be responsible for its altered biological activity (*Id.*). Shima et al., conclude that "HGF and dHGF should be distinguished from each other to avoid confusion caused by their different biological actions." (*See 1st paragraph on page 812).*

Similarly, Kinosaki et al., (published in 1998, **Exhibit C**) report that dHGF and HGF are distinguishable and that residues on HGF that are essential for exerting its biological activity are not the same as those found on dHGF (*see abstract*). Further, Otsuka et al. (published in 2000, **Exhibit F**) establish that other splicing variants of HGF (NK1 and NK2) have drastically different biological properties and, in fact, antagonize HGF activity. The authors state that "historically, defining the biological activities associated with these variants [HGF] has been

somewhat elusive and a point of contention in the field." *(See last paragraph, page 2055).* The authors also note that the in vitro biological activities of HGF variants are context dependent and greatly influenced by the target cell and culture conditions, which "stand as a testament to the requirement for in vivo models to access their bona fide activities." *(See 1st paragraph, page 2056).*

Similarly, Lindsey and Brenner (published in 2002, **Exhibit D**), report the finding of dNK1 and dNK2, more splice variants of HGF and suggest that the deletion in the first kringle domain may impact heparin binding and localization to heparinized regions as well as having an impact on the binding of the molecules to other proteins, for example, proteins of the extracellular matrix. *(See last paragraph, page 85 and first paragraph page 86).*

Additionally, Ozeki and Tabata (published in 2006, **Exhibit E**), report that dHGF was much more rapidly released from gelatin hydrogel than HGF indicating that dHGF has much more reduced affinity to gelatin (i.e., collagen). *(See abstract).* The authors confirm that dHGF and HGF have structural differences *(see 2nd paragraph, page 140)* and discover that dHGF and HGF have significantly different biological properties, namely the ability to bind acidic gelatin. Interestingly, the authors also report that HGF affixed to hydrogel had much greater resistance to protease degradation; whereas dHGF diffused from the gel and was rapidly degraded *(see second paragraph, page 149 and Figure 4).*

Accordingly, **Exhibits B-F,** which were all published after Nakamura et al. (EP 461,560A1) and Seki et al., establish that dHGF is structurally and functionally different than HGF. Furthermore, the references underscore the unpredictable nature of the HGF variants and, in fact, teach away from using HGF variants, in particular dHGF, to promote granulation and wound healing. As reported by Shima et al (**Exhibit B**), dHGF is more than 70 fold less soluble than HGF; and as reported by Lindsey and Brenner (**Exhibit D**), dHGF is less likely to interact with proteins of the extracellular matrix than HGF; and as reported by Ozeki and Tabata (**Exhibit E**) dHGF is readily dissociated from collagen and is more sensitive to protease digestion than HGF when formulated in a hydrogel. In view of these references, one of skill in the art would not reasonably believe that HGF and dHGF are structurally and functionally equivalent and would not have a reasonable expectation that a topically administered dHGF would promote granulation formation and/or wound healing.

The Examiner has also cited Nakamura et al (US5,342,831) for the proposition that topical wound healing with HGF formulations was known and that one would readily swap dHGF for HGF in the formulations described therein. Again, Applicants point-out that the state of the art at the time of filing the present application established that dHGF and HGF were not interchangeable, that HGF variants, especially dHGF, were art recognized to have vastly different structures, functions, and physicochemical properties and that one would not be motivated to topically apply dHGF based on its poor solubility compared to HGF and its reduced interaction with extracellular matrix proteins, in particular, gelatins used in hydrogel formulations.

The Examiner has also cited Yoshida et al., for the proposition that antibodies directed to HGF inhibited granulation formation. Applicants respectfully submit that this reference only shows the relationship between HGF and granulation formation in vivo and does not support a finding that topically applied HGF, much less topically applied dHGF, can promote granulation formation and wound healing.

The Examiner has also cited Morishita et al (US 7,247,620) or Morishita et al. (WO 02/089854) for the proposition that diabetic skin ulcers can be treated by topical administration of an HGF gene to promote granulation and that in light of the other references cited, topical administration of dHGF to promote granulation would be obvious. As illustrated in Toyoda et al., over expression of the HGF gene in skin cells results in a very different biological response than topical application of HGF protein. Further, Morishita et al., teach away from topical administration of HGF protein. Morishita et al state, for example, that attempts have been made to use HGF in pharmaceutical agents but HGF's short half-life makes maintenance of therapeutic concentrations in the blood difficult and therefore translocation of an effective HGF dose to an affected area is problematic. *(See Column 2*, lines 26-33*)*. Morishita et al. also state:

> Proteinaceous formulations are generally administered intravenously. HGF has been administered in ischemic disease models both intravenously and intra-arterially (Circulation 97: 381-390(1998)). Such intravenous or intra-arterial administrations of HGF to animal models have revealed HGF's effectiveness on ischemic or arterial diseases. However, as yet, no conclusion has been reached with regard to a specific and effective method for administration, effective dose, and such. This is particularly so in the case of the HGF protein, due to the above-mentioned problems with half-life and transfer to the affected area. Thus, to date

there has been no conclusion regarding an effective method of administration, effective dose, etc. (*See Column 3, lines 64-67 and Column 4, lines 1-9).*

Applicants respectfully submit that Morishita et al., supports the fact that HGF gene therapy is significantly different than HGF protein–based therapy and that based on the excerpts provided above, one of skill in the art would not believe that the results seen in HGF gene therapy are reasonably predictive of the results one would observe with HGF protein-based therapy, much less with dHGF protein-based therapy.

In summary, Applicants submit that the cited references establish that there is a relationship between HGF and granulation promotion in skin, that HGF has been used to treat skin ulcers, and that many HGF variants having vastly different structural and biological properties, including dHGF, were known. However, the nexus between topical application of HGF and granulation formation is not reasonably predictive based on transgenic HGF models that show an opposite biological response to that seen with HGF protein administration, antibody inhibition experiments showing a decrease in granulation when HGF is inhibited, and HGF gene therapy approaches that disclose that administration of HGF protein is problematic, troubled by a short-half life and ineffective translocation to the affected area. Even further removed is the unexpected finding that topical administration of dHGF can promote granulation formation and wound healing given that the molecule is 70 times less soluble than HGF, has a different tertiary structure than HGF, has different biological properties than HGF, has less affinity for extracellular matrix proteins than HGF, and is more prone to protease digestion than HGF in hydrogel preparations. Despite the drawbacks to topical application of dHGF, Applicants have unexpectedly found that that among all of the HGF variants, dHGF is excellent in promoting granulation formation, neovascularization, and wound healing when it is topically administered and that dHGF is particularly suited for treating skin ulcers (*see* [0026]). Accordingly, Applicants respectfully request that the rejections under 35 USC sec. 103(a) be withdrawn.

CONCLUSION

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

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HEPATOCYTE GROWTH FACTOR AND ITS VARIANT WITH A DELETION OF FIVE AMINO ACIDS ARE DISTINGUISHABLE IN THEIR BIOLOGICAL ACTIVITY AND TERTIARY STRUCTURE

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Summary: A naturally occurring splice variant of hepatocyte growth factor (HGF) lacks a 5-amino acid sequence in the first kringle domain. Comparison of HGF and the deletion variant (dHGF) revealed that the deletion significantly altered the biological activities, solubility, and immunological property of HGF. HGF was respectively about 20-, 10-, and 2-fold more potent than dHGF in the stimulation of DNA synthesis in human umbilical vein endothelial cells, human aorta smooth muscle cells, and NSF-60 (murine myeloblastic cells). Conversely, dHGF was respectively about 3-, 2-, and 2-fold more potent than HGF in the stimulation of DNA synthesis in LLC-PK1 (pig kidney epithelial cells), OK (American opossum kidney epithelial cells), and rat hepatocytes. Moreover, HGF was over 70-fold more soluble than dHGF in PBS. Several monoclonal antibodies raised against dHGF recognized only dHGF and neither HGF nor reduced dHGF, demonstrating that the deletion caused a tertiary structural change. The structural change in HGF may be responsible for its altered biological activities and solubility.

Hepatocyte growth factor (HGF) (1,2), also designated as scatter factor (SF) (3) or fibroblast-derived tumor cytotoxic factor (F-TCF) (4,5), is a heparin-binding basic protein with an approximate molecular mass of 80 kD. HGF is a disulfide-linked heterodimer composed of an α-chain of 52-56 kD and a β-chain of 30-34 kD (1,2). Recent studies have revealed that HGF not only functions as a mitogen for hepatocytes, it also stimulates the growth of various epithelial (6,7) and endothelial cells(6,8); it inhibits the growth of tumor cells (4,8,9); and it stimulates the mobility of epithelial (3) and endothelial cells (10). The nucleotide sequence analysis of cDNA clones for human HGF (11,12) predicted that HGF consists of 6 major domains: a hairpin and four kringles in the α-chain, and a serine protease-like domain in the β-chain.

In addition to the originally reported cDNA, another major variant which lacks 15 nucleotides encoding a 5-amino acid residue (the FLPSS sequence) in the first kringle domain, has been

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isolated (5,6,13). The significance of the existence of the deleted form of HGF (dHGF), however, remains to be established. Structure-function studies with partially truncated HGF molecules (14,15) have demonstrated that N-terminal three domains in the α-chain of HGF (the hairpin and the first two kringles) are essential for the biological function of HGF. This implies that the deletion of 5 amino acids in the first kringle might affect the biological activity of HGF. Our preliminary result indicated that dHGF had higher mitogenic activity than HGF for rat hepatocytes (5), but another study reported that there was no difference between the biological activities of the two HGFs (13). To elucidate whether the roles of these two forms of HGF are the same, we made detailed analyses of the physicochemical, immunological, and biological properties of HGF and dHGF using highly purified recombinant proteins.

MATERIALS AND METHODS

Expression of HGF and dHGF cDNAs. Plasmid to express HGF or dHGF was constructed by inserting a 2.3-kb fragment of HGF or dHGF cDNA (5) into pcDNAI (Invitrogen) under cytomegarovirus promoter, and a 2.4-kb fragment of a mouse dihydrofolate reductase (DHFR) transcription unit from pAdD26SVA (16) into the unique NheI site of pcDNAI. The expression plasmid (10μg) and pSV2 neo (1μg) (17) were co-transfected into Namalwa cells by the liposome-mediated transfection method (18). The transformed Namalwa cells were selected with G418 and subsequently gene-amplified with methotrexate. Clones were screened for high HGF or dHGF production by an ELISA using monoclonal antibodies that recognize both HGFs equally (19).

Target cells. Adult rat hepatocytes were prepared by the method of Seglen (20). Human umbilical vein endothelial cells, HUVEC, and human aorta smooth muscle cells, AOSMC, were purchased from Kurabo Co. (Japan). NFS-60 (murine myeloblastic cells) was kindly provided by Dr. T. Suda (Kumamoto University, Japan). LLC-PK1 (pig kidney epithelial cells) and OK (American opossum kidney epithelial cells) were purchased from American Type Culture Collection (ATCC, USA).

Preparation of highly purified HGF and dHGF. Namalwa cells producing HGF or dHGF were cultured in DMEM (Gibco) with 5% calf serum at 37°C for 7 days. HGFs in the conditioned media were purified by a combination of CM sepharose, Heparin CL-6B, Mono S-HPLC, and Heparin 5PW-HPLC, as described previously (5). The protein concentration was determined by the method of Lowry using bovine serum albumin as a standard protein. For biological assays, the HGFs were diluted in PBS containing 0.25% human serum albumin and 0.001% Tween 80, and were sterilized by filtration through a 0.22-μm membrane filter. For physicochemical studies, the HGFs were diluted in PBS containing 0.01% Tween 20. The concentration of each HGF in the solution was reconfirmed by the ELISA as described above.

Cell culture and assay for cell growth. Adult rat hepatocytes suspended in William's E medium (Gibco) with 10 % fetal bovine serum (FBS) and 10 nM dexamethasone were inoculated at an initial cell density of 10^4 cells /50μl /well in 96-well plates and were incubated at 37°C for 24 hours. HUVE cells were grown in E-GM UV medium (Kurabo). The trypsinized cells were suspended in Medium 199 (Gibco) with 10% FBS and were inoculated at a cell density of 5×10^3 cells /50μl /well. AOSM cells were grown in S-GM (Kurabo) . The trypsinized cells suspended in S-BM (Kurabo) with 5% FBS were inoculated at a cell density of 10^4 cells /50 μl /well and were incubated at 37°C for 48 hours. LLC-PK1 cells were maintained in DMEM with 10% FBS. The cells suspended in serum-free DMEM were inoculated at a cell density of 10^4 cells /50 μl /well and were incubated at 37°C for 48 hours. NFS-60 cells were maintained in RPMI 1640 (Gibco) with 10% FBS. The cells suspended in the same medium were inoculated at a cell density of 5×10^3 cells /50 μl /well. A serially diluted HGF or dHGF in the respective medium was added to each well of the cell cultures (50 μl /well). The plates were then incubated at 37°C for 24 hours.

Subsequently, 1 μCi /10 μl of [methyl-^{3}H]thymidine (Amersham, UK; 85Ci/mmol for rat hepatocytes and 5Ci/mmol for the other cells) was added to each well and the plates were further incubated for 2 hours. The culture plates for anchorage-dependent cells were washed with PBS (200 μl /well) and trypsinized. The radioactivity incorporated into the cells was determined using a Direct Beta Counter MATRIX 96 (Packard, USA). Data are presented as the mean ± SD of triplicate cultures. Each experiment was repeated by using at least two separate preparations of the HGFs.

Solubility test. The purified HGFs were dialyzed against water and lyophilized. The lyophilized HGF and dHGF were suspended in PBS containing 0.01% Tween 20 at 37°C and were agitated for 30 minutes. After centrifugation at 30,000 x g for 30 minutes at 5°C, the concentration of protein in the supernatant was determined by the method of Lowry.

Selection of monoclonal antibodies against HGF or dHGF. Monoclonal antibodies were prepared by the published method (21) with our modifications (19). Briefly, BALB/c mice were immunized intraperitoneally with 100 μg of purified dHGF. Splenocytes from the immunized mice were fused with P3X63-Ag8.653 mouse myeloma cells (ATCC, CRL1580). The specificity of each monoclonal antibody was determined by using a solid phase ELISA as follows. A 96-well plate (MaxiSorp, Nunc, Denmark) was coated with HGF, dHGF, or reduced dHGF (1μg/well). Subsequently, the plate was filled with 50% Block Ace (Snow Brand Milk Products, Japan) and incubated for 1 hour at room temperature. After the plate was washed 3 times with PBS containing 0.1% Tween 20, test samples (hybridoma culture medium or purified monoclonal IgG solution) diluted in 0.2 M Tris HCl, pH 7.3, containing 50% Block Ace and 0.1% Tween 20, were applied to the plate, followed by incubation for 3 hours at 37°C. After washing, the amount of the bound antibody was detected with peroxidase-linked goat anti-mouse IgG (Cappel, Belgium). Construction of a sandwich ELISA was performed according to the procedure as described (19).

RESULTS AND DISCUSSION

Purity and molecular weight of the two HGFs. For initial characterization of the two HGFs, we analyzed the purity and molecular weight of the purified HGFs. The two HGFs were subjected to SDS-PAGE under reducing and non-reducing conditions. Each HGF showed homogeneous electrophoresis bands with purity of over 99%, and the band sizes were as predicted (data not shown). The two HGFs were next applied to a high performance gel chromatography column (Superose 12/30, Pharmacia) equilibrated with PBS containing 0.01% Tween 20. Each HGF showed the same profile with a single peak, and the molecular mass of each HGF was calculated to be 74 kD, indicating that each HGF dissolved as a monomer in the aqueous solution.

Difference between the two HGFs in biological activity. To clarify the difference between the two HGFs, we compared the biological activity of each HGF. Dose-response curves for the stimulation of DNA synthesis in rat hepatocytes by HGF and dHGF were very similar up to about 10 ng/ml, but were different significantly at higher concentrations (Fig. 1). HGF markedly decreased its activity in a tested dose range of 10 to 500 ng/ml, while dHGF gave the maximal activity in the same dose range. Specific activity of dHGF was maximally 1.9-fold higher than that of HGF in that dose range. We observed the same phenomena as seen in this experiment when the cDNAs were expressed in CHO cells (5) and in C127 cells (data not shown). Matsumoto et al. (14) showed the same result in the dose-response curves between the two HGFs, which are expressed transiently in COS cells. These results indicate that the difference does not depend on expression

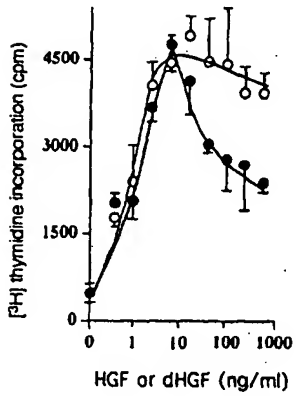

Fig. 1. Effect of HGF or dHGF on the stimulation of DNA synthesis in rat hepatocytes. Hepatocytes were cultured with HGF (closed circles) or dHGF (open circles). Results are presented as the mean ± SD of triplicate cultures.

systems but is due to the deletion of 5 amino acids (FPLSS) in the first kringle of HGF. Seki et al. (13), however, have reported that specific activities of HGF and dHGF transiently expressed in COS cells are almost the same. It is likely that the dosages of both materials in that experiment were in the dose range (< 10 ng/ml) in which both materials show almost the same specific activities. The biological difference observed at higher concentrations (>10 ng/ml) implies that the mechanisms for the stimulation of DNA synthesis are probably different between HGF and dHGF.

In addition to the difference between the two HGFs in the stimulation of hepatocytes, marked differences between the biological activities of the two HGFs were observed in the other target cells. HGF was more potent than dHGF in the stimulation of DNA synthesis in mesenchymal cells, such as HUVEC, AOSMC, and NFS-60 (Fig. 2A, B, and C), although dHGF was more potent than HGF in the stimulation of DNA synthesis in epithelial cells, such as LLC-PK1 and OK (Fig. 2D and E). To evaluate the difference in the growth-stimulating potency of the two HGFs on various types of cells, we compared a half-maximal dose of HGF and a dose of dHGF that gave the same level of activity as the half-maximal activity of HGF. HGF was respectively about 20-, 10-, and 2-fold more potent than dHGF in the growth stimulation of HUVEC, AOSMC, and NFS-60, and dHGF was respectively about 3- and 2-fold more potent than HGF in LLC-PK1 and OK cells. dHGF may be more specific for the growth of epithelial cells than HGF, and the two HGFs may play different roles in physiological actions.

The difference between the biological activities of the two HGFs demonstrated here may account for discrepancies in the results of past experiments using HGF. It was reported that HGF acts only on epithelial cells and not mesenchymal cells (7). On the other hand, other studies have shown that HGF acts as a mitogen for various mesenchymal cells, such as melanocytes, endothelial cells, and myeloblastic cells, NFS-60 (22). In addition to these observations, we have found that HGF acts

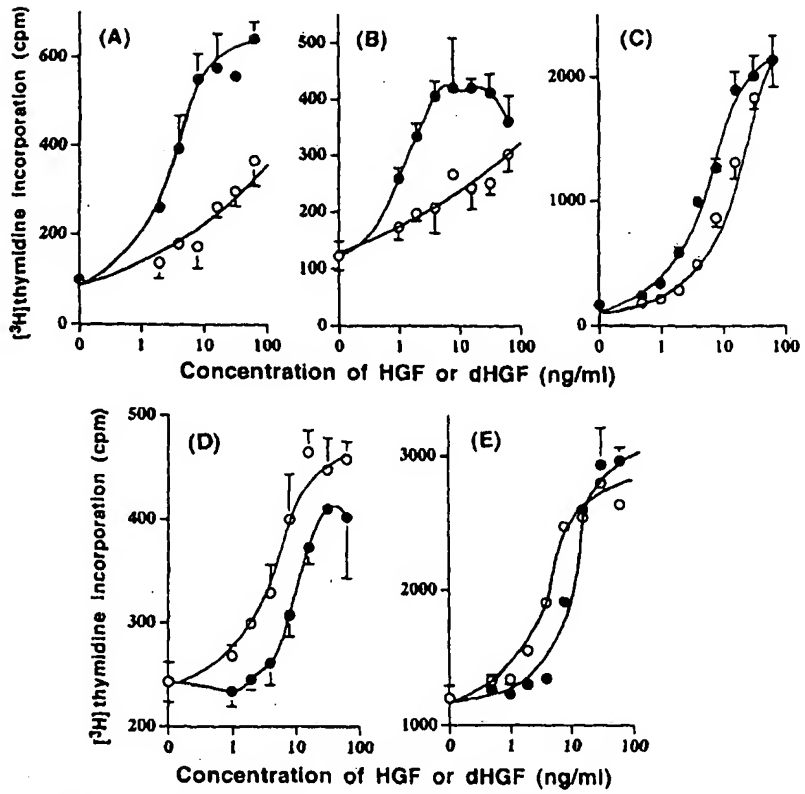

Fig. 2. Effect of HGF or dHGF on the stimulation of DNA synthesis in various kinds of mesenchymal cells (A, B, and C) and epithelial cells (D and E). Cells were cultured with HGF (closed circles) or dHGF (open circles). Results are presented as the mean ± SD of triplicate cultures. (A) human umbilical vein endothelial cells; HUVEC, (B) human aorta smooth muscle cells; AOSMC, (C) NFS-60 (murine myeloblastic cells), (D) LLC-PK1 (pig kidney epithelial cells), (E) OK (American opossum kidney epithelial cells).

as a mitogen for human smooth muscle cells, AOSMC, although no mitogenic effect of HGF on a rat AOSM cell line had been previously reported (23). Our results therefore suggest that HGF and dHGF should be distinguished from each other in order to avoid confusion caused by their different biological actions.

Difference between the two HGFs in physicochemical property. We next compared the physicochemical properties of the two HGFs. There was no significant difference between the two HGFs in sialic acid contents, N-linked sugar structure, and heat- and pH-stability (data not shown). A marked difference in solubility, however, was observed between the two HGFs. dHGF was soluble in PBS up to 1.34 mg /ml. In contrast, HGF was soluble over 90 mg /ml under the same conditions. Since our preliminary experiment indicated that the presence of NaCl (greater than 150 mM) affects the solubility of the HGFs, the concentration of sodium ion in the lyophilized HGFs was determined by an atomic absorption analysis. The calculated NaCl concentrations in the

HGF and dHGF solutions at a concentration of 1 mg protein per ml water were 0.55 and 0.94 μM, respectively, indicating the absence of any contribution of NaCl from the lyophilized materials in the solubility test.

Difference between the two HGFs in tertiary structure. A marked difference in solubility suggests that the deletion in the first kringle affects the tertiary structure of HGF. To demonstrate that the deletion causes a conformational change, we screened dHGF specific monoclonal antibodies by a solid phase ELISA. The recognition of antigens varied depending on the antibodies used. P1C8, P2D6, and B4A2 recognized HGF and dHGF equally, but they did not recognize dHGF reduced with 2- mercaptoethanol. D3B3 recognized both HGF and dHGF, but it recognized HGF to a lesser extent. A2G9 and H9E3 recognized dHGF but not HGF or reduced dHGF (Table 1). The specificity of antibodies was confirmed by a sandwich ELISA. The combination of P1C8 and peroxidase-linked P2D6 detected HGF and dHGF equally (Fig. 3A). On the other hand, the combination of H9E3 and peroxidase-linked P2D6 detected only dHGF (Fig. 3B). Moreover, we observed that these dHGF specific antibodies recognized a mutant dHGF with no N-linked oligosaccharide chains as well as the wild-type dHGF (data not shown). These findings demonstrate that dHGF specific antibodies recognize three-dimensional structures newly formed in the protein moiety by the deletion of 5 amino acids. Thus, we concluded that HGF and dHGF are different in tertiary structure. The structural change in HGF therefore may alter its biological activities and solubility.

The c-Met transmembrane protein is now known to be a functional receptor for HGF (24,25). Recent studies, however, suggest that other receptors may also be present. Several isoforms of the c-Met receptor generated by alternative splicing have been reported (26). A Scatchard analysis (24) and cross-linking study (27) suggested the presence of two classes of high affinity binding sites for HGF. Novel putative tyrosine kinase receptors of the Met family have also been reported (28-30).

Table 1. Antigen specificity of monoclonal antibodies

Antibodies	Maximal antibody binding (OD 492 nm) Antigen		
	dHGF	reduced dHGF	HGF
P1C8	1.21	0.03	1.19
P2D6	1.23	0.03	1.28
B4A2	1.02	0.03	1.04
D3B3	1.31	0.07	0.56
A2G9	1.42	0.02	0.02
H9E3	1.21	0.09	0.09

Blank value was 0.03 OD at 492 nm.
Results are presented as the mean of duplicate experiments.

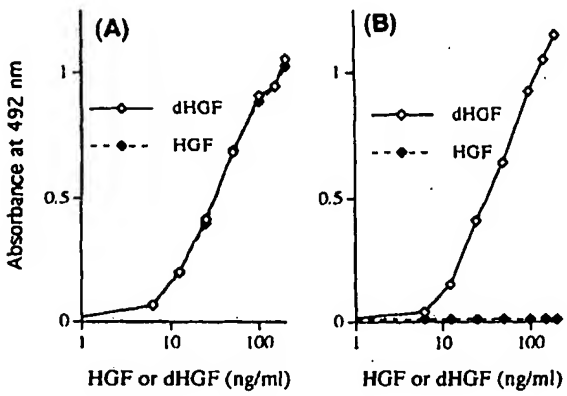

Fig. 3. Standard curves of ELISA for HGF and dHGF obtained at different combinations of monoclonal antibodies. (A) Combination of P1C8 and P2D6 monoclonal antibodies. (B) Combination of H9E3 and P2D6 monoclonal antibodies.

Thus, the presence of receptors other than the c-Met receptor may be a possible explanation for the difference between the two HGFs in their target cell specificity demonstrated in the present study. The structural change caused by the deletion of 5 amino acids in the first kringle may alter the binding of the HGFs to a receptor, since the kringle structure is well known to play a role in protein-protein interaction. Further study of HGF receptors, including intracellular signaling cascades, should facilitate understanding of the difference between the biological activities of the two HGFs.

ACKNOWLEDGMENTS

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EXHIBIT C

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Analysis of deleted variant of hepatocyte growth factor by alanine scanning mutagenesis: identification of residues essential for its biological function and generation of mutants with enhanced mitogenic activity on rat hepatocytes

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Abstract To understand the structure-function relationship of hepatocyte growth factor (HGF) in more detail, we analyzed one of the other forms of HGF, deleted variant of HGF (dHGF), by alanine scanning mutagenesis. We show here that there are at least four sites important for dHGF to stimulate DNA synthesis in cultured adult rat hepatocytes, and that the residues of HGF essential for exerting its biological activity are not identical to those of dHGF. In addition, two mutants showed a decrease (approximately three-fold) in EC_{50} compared with wild-type dHGF in an assay of mitogenic activity on rat hepatocytes.

Key words: Hepatocyte growth factor; Deleted variant of hepatocyte growth factor; Alanine scanning mutagenesis

1. Introduction

Hepatocyte growth factor (HGF) is a heparin binding basic protein initially identified as a potent mitogen for hepatocytes [1–3]. The molecular cloning of HGF cDNA has predicted its primary and tertiary structures [4,5]. Subsequently, both scatter factor and fibroblast-derived tumor cytotoxic factor were found to be identical to HGF [6–10].

Recent studies have revealed that HGF is biologically multifunctional. It is a mitogen not only for hepatocytes but also for various epithelial cells [11–14] and endothelial cells [9,13–15], whereas it suppresses the growth of tumor cells [7,9,13,14]. HGF executes these activities through its receptor, the c-*met* proto-oncogene product [13,14,16–18].

HGF is a disulfide-linked heterodimer composed of an α chain with a molecular mass of 52–56 kDa and a β chain of 30–34 kDa [19,20]. HGF consists of six major domains: the N-terminal domain (including the hairpin loop) and four kringle domains in the α chain, and a serine protease-like domain in the β chain [4,5].

A naturally occurring HGF variant (deleted variant of hepatocyte growth factor, dHGF) that arises from an alternatively spliced transcript and lacks five amino acids (Phe-Leu-Pro-Ser-Ser) in the first kringle domain was also found in many cells or cell lines [10,21]. It has been reported that dHGF is distinguishable from HGF in biological activity and tertiary structure [10,22,23].

Numerous HGF mutants have been prepared in investigations of the HGF structure-function relationship [22,24–28]. Through these studies, it has been established that the N-terminal domain and the first and second kringle domains are required for its biological functions [22,24–26]. The importance of the first two domains has been demonstrated by the finding that a variant consisting of the N-terminal domain and the first kringle domain binds to the receptor and has a low level of scatter activity [27]. More recently, a series of point mutations were systematically introduced into the first two domains of HGF to identify the residues essential for the mitogenic activity on rat hepatocytes [28]. Of more than 50 HGF mutants constructed in many laboratories, no mutant has been found to have specific activity significantly higher (two-fold or more) than that of HGF.

Since no structure-function study has been carried out with dHGF, we conducted a mutational analysis of dHGF. To analyze the role of the residues in the two N-terminal domains, alanine scanning mutagenesis was employed [29]. We describe here the identification of the residues of dHGF required for stimulating DNA synthesis in hepatocytes. We also show that these residues are not identical to those of HGF. In addition, two mutants showed a decrease in EC_{50} (dose required for half-maximal cell proliferative response) in an assay of mitogenic activity on rat hepatocytes. The two dHGF mutants are, therefore, the most potent mitogen for hepatocytes among the HGF and dHGF variants constructed to date.

2. Materials and methods

2.1. Cells

Adult rat hepatocytes were prepared by the method of Seglen [30]. NFS-60 cells (murine myeloblastic cells) were kindly provided by Dr. T. Suda (Kumamoto University, Japan). OK cells (American opossum kidney epithelial cells) and Chinese hamster ovary (CHO) cells were purchased from American Type Culture Collection (ATCC).

2.2. Construction of expression vectors for dHGF and its mutants

The plasmid to express human dHGF, designated pSRαTCF, was constructed as follows. An SR α promoter-based vector termed pcDL-SR α 296 [31] (a gift from Dr. Y. Takebe) was digested with restriction enzymes *Pst*I and *Kpn*I, and the ends were blunted using DNA blunting kit (Takara Shuzo). dHGF cDNA fragment was excised from pUCTCF, a plasmid with the entire coding sequence for

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Abbreviations: dHGF, deleted variant of hepatocyte growth factor; HGF, hepatocyte growth factor; ELISA, enzyme-linked immunosorbent assay; IMDM, Iscove's modified Dulbecco's medium; FPLC, fast protein liquid chromatography; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; EC_{50}, dose required for 50% maximal cell proliferative response

PII: S0014-5793(98)00971-5

human dHGF inserted between the *Bam*HI and *Sph*I sites of pUC 18 (Takara Shuzo), by digesting with *Bam*HI and *Sph*I. The fragment was then treated with the DNA blunting kit, and inserted into the blunt-ended pcDL-SR α296 vector. Site-directed mutagenesis was carried out by a recombinant polymerase chain reaction (PCR) technique as described by Higuchi [32]. pUCTCF was used as a template for the PCRs. A *Bst*PI/*Eco*RV fragment of pSRαTCF, which encodes the N-terminal region and the first kringle domain, was replaced with the *Bst*PI/*Eco*RV fragment derived from each PCR product to generate the various vectors to express dHGF mutants. CHO cells were transfected with a mixture of 200 μg of each expression plasmid and 10 μg of pSV2bsr, an expression vector for blasticidin S resistant gene (Funakoshi), by electroporation, and cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS at 37°C. After 3 days of incubation, the cells suspended in IMDM containing 10% FBS and 5 μg/ml of blasticidin S (Funakoshi) were seeded at an initial density of 10^4 cells/200 μl/well in 96-well plates. Two weeks after the inoculation, the amount of mutant dHGF in the cultured medium in each well was measured by an ELISA as previously described [33], and the clones which expressed mutant proteins were selected.

2.3. Nomenclature of mutants

The mutations or mutants are defined by the following, in the order shown: (a) residue(s) to be replaced, (b) the position of the replaced amino acid or the first residues of the mutated sequences in the amino acid sequence, and (c) the residue or the sequence generated by the mutagenesis (e.g. R42A or KIKTKK27AIATAA). The position of each mutation is described by indicating the residue number of the first residue of the cluster in the amino acid sequence and the original sequence (e.g. 42R or 27KIKTKK). Amino acid residues are numbered starting at the amino-terminus of the mature protein. The deduced amino acid sequences of HGF and dHGF together with the positions of the mutations in dHGF mutants are shown in Fig. 1.

2.4. Heparin Sepharose chromatography

The affinity of wild-type and dHGF mutants for heparin was determined by FPLC on a HiTrap heparin column (Pharmacia). Conditioned medium containing each dHGF mutant was applied to the column (1 ml) equilibrated with 20 mM Tris-HCl (pH 7.5)-0.01% Tween 80 (Sigma). The protein was eluted with a 45 min linear gradient of 0–1.5 M NaCl in the buffer at a flow rate of 0.5 ml/min, and fractions (0.5 ml) were collected. The concentration of the dHGF mutant in each fraction was determined by an ELISA employing a rabbit anti-dHGF polyclonal antibody.

2.5. Purification

CHO cells producing dHGF mutants were cultured in IMDM with 5% calf serum at 37°C for 1 week. Each mutant in the conditioned medium was purified as previously described [23] with the following modification. The conditioned medium was applied to an S-Sepharose column (25×80 mm, Pharmacia) at a flow rate of 4 ml/min. The column was washed with an equilibration buffer (10 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl and 0.01% Tween 20) and then eluted with the buffer containing 2 M NaCl. The fractions containing the mutant were pooled and dialyzed against 10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl and 0.01% Tween 80. The mutants were further purified to homogeneity by the combination of Mono S (Pharmacia) and heparin-5PW FPLC (Tosoh) as previously described [23]. The protein concentration was determined by the method of Lowry using bovine serum albumin as a standard protein.

2.6. Biological assays

Adult rat hepatocytes suspended in William's E medium containing 10% FBS and 10 mM dexamethasone were seeded at an initial cell density of 10^4 cells/100 μl/well in 96-well plates. The cells were incubated for 24 h at 37°C. dHGF and dHGF mutants were partially purified with heparin Sepharose, resuspended in phosphate-buffered saline (PBS) containing 0.01% Tween 80 and concentrated with Centricut (Kurabo). Each dHGF mutant was serially diluted with the medium and added to each well. The plates were incubated for 22 h at 37°C. Subsequently, 1 μCi/10 μl of [*methyl*-^{3}H]thymidine (85 Ci/mmol, Amersham) was added to each well and the plates were further incubated for 2 h. The cells were washed with PBS and then trypsinized. The radioactivity in the wells was measured using a Direct Beta Counter, Matrix 96 (Packard). OK cells were maintained in DMEM containing 10% FBS. The cells suspended in the medium were seeded at a cell density of 10^4 cells/100 μl/well and were incubated at 37°C for 24 h. The medium was replaced with 100 μl of fresh serum-free DMEM and the cells were further incubated for 48 h. Subsequently, the medium was replaced with 50 μl of fresh serum-free DMEM, and then serially diluted dHGF or each mutant in the serum-free medium containing 0.1% bovine serum albumin was added to each well of the plates (50 μl/well). After the incubation at 37°C for 22 h, 1 μCi/10 μl of [*methyl*-^{3}H]thymidine (5 Ci/nmol, Amersham) was added to each well and the plates were further incubated for 2 h. The cells were washed with PBS and trypsinized. The radioactivity incorporated into the cells was determined using Matrix 96. NFS-60 cells were maintained in RPMI 1640 (Life Technologies) containing 10% FBS and 10% conditioned medium of WEHI-3 cells. The cells suspended in RPMI 1640 with 10% FBS were seeded at a cell density of 10^4 cells/50 μl/well. Serially diluted dHGF or each mutant was added to each well (50 μl/well), and the plates were further incubated. Twenty-four hours later, 10 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well and the plates were further incubated for 4 h. Subsequently, 100 μl of 10% SDS in 0.01 M ammonium chloride was added to each well. The plate was then incubated overnight at 37°C to solubilize the cells. The optical density at 590 nm of each well of the plates was measured.

3. Results

3.1. Identification of residues essential for the biological function of dHGF by alanine scanning mutagenesis

Mutants were produced in which multiple or single charged residue(s) in the N-terminal domain and the first kringle domain was/were replaced with alanine, and their mitogenic activity was determined. The specific activity of the mutants to stimulate DNA synthesis in rat hepatocytes at a concentration of 1.25 ng/ml is shown in Fig. 2A. The results are expressed as activity relative to that of wild-type dHGF. Of the N-terminal domain mutants, DKARK59AAAAA and ENKD89ANAA were inactive. Next, mutants in which each of the charged residues in 59DKARK and 89ENKD was replaced with alanine (with an exception of RK62AA, where both 62R and 63K were replaced with alanine residues) were generated. As shown in the left two columns in Fig. 2B, all six mutants maintained the biological activity, suggesting that individual residues are not functionally crucial. In contrast, none of the

```
                N-terminal basic region
HGF    1:QRKRRNTIHEFKKSAKTTLIKIDPALKIKTKKVNTADQCANRCTRNKG 48
dHGF   1:QRKRRNTIHEFKKSAKTTLIKIDPALKIKTKKVNTADQCANRCTRNKG 48
         AAAA  A     A   AIA   AIATAA    A   A  A A
         2     9     16  21    27        37  42 4547

HGF   49:LPFTCKAFVFDKARKQCLWFPFNSMSSGVKKEFGHEFDLYENKDYIRN 96
dHGF  49:LPFTCKAFVFDKARKQCLNFPFNSMSSGVKKEFGHEFDLYENKDYIRN 96
            A   AAAAA              AAA  AAFA AHAA  A
            54  59                 78   83   89    95


          First kringle domain
HGF   97:CIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEHSFLPSSYRGKDLQENY 145
dHGF  97:CIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEHS-----YRGKDLQENY 140
            AGA         A  A        AAA       AGAA  A
            101         113 117      127       132   138

HGF  146:CRNPRGEEGGPWCFTSNPEVRYEVCDIPQCSEVE              179
dHGF 141:CRNPRGEEGGPWCFTSNPEVRYEVCDIPQCSEVE              174
         A AGAA         AVAYA A    AVA
         142145         159   166  172
```

Fig. 1. Alignments of the amino acid sequences of human HGF and dHGF (excluding the signal peptide). The amino acids are numbered starting at the amino-termini of the mature proteins. Positions of alanine substitutions in dHGF mutants and their sequences after mutagenesis are shown in the bottom row.

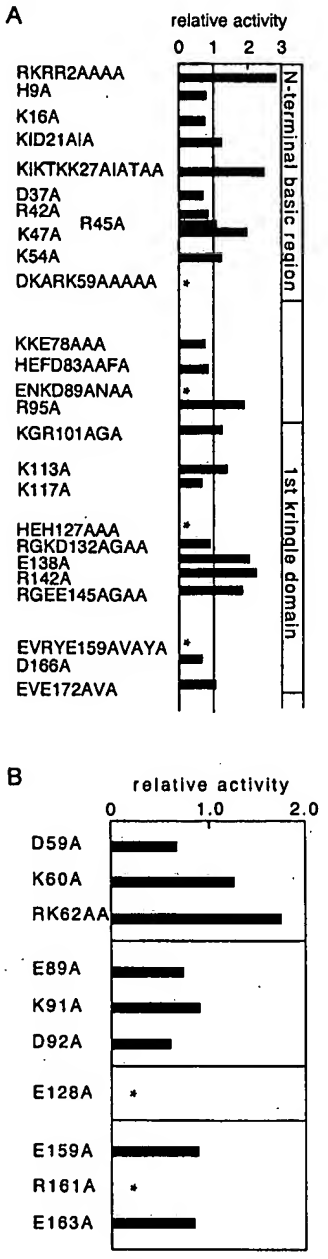

Fig. 2. Biological activity of alanine substitution mutants relative to dHGF. A: Biological activity of the alanine substitution dHGF mutants relative to dHGF. Asterisks indicate lack of detectable activity. B: Relative biological activity of dHGF mutants with single or double (62RK) substitution(s) in the four mutation-sensitive regions identified by the experiment shown in A. Asterisks indicate lack of detectable activity.

mutations in the hairpin loop structure, D37A, R42A, R45A, K47A and K54A, markedly affected the biological activity (Fig. 2A). The mutation at two sites in the first kringle domain, HEH127AAA and EVRYE159AVAYA, abolished the biological activity. To determine the residues essential for the activity, a single amino acid substitution was introduced into these two 'mutation-sensitive' sites. As shown in the right two columns in Fig. 2B, two mutants, E128A and R161A, were inactive, indicating that the residues 128E and 161R are required for dHGF to exert its biological activity.

As shown in Fig. 2A, several mutants were found to be more potent than dHGF in stimulating DNA synthesis in rat hepatocytes. These mutants were purified to homogeneity (purity was over 95%) and their biological activity was evaluated. In this second screening, two mutants, RKRR2AAAA and KIKTKK27AIATAA, reproducibly showed increases in specific activity (see below) and were characterized further. Mutants RKRR2AAAA and KIKTKK27AIATAA were renamed #2 and #27, respectively.

3.2. *Stimulation of DNA synthesis in adult rat hepatocytes by purified mutants #2 and #27*

Fig. 3 shows the ability of the two mutants to stimulate DNA synthesis in cultured adult rat hepatocytes. In a dose range of 1–16 ng/ml, the two mutants were significantly higher in specific activity than dHGF. The EC_{50} values for #2 and #27 were approximately three-fold lower than that for dHGF. The maximum stimulation levels for #2 and #27 were the same as that for dHGF.

3.3. *Biological activities of mutants #2 and #27 on NFS-60 and OK cells*

As shown in Fig. 4A, the two mutants were less potent than dHGF in the stimulation of NFS-60 (a mouse bone marrow cell line) cell proliferation in a dose range of 0.5 to 32 ng/ml. Mutants #2 and #27 showed increases in EC_{50} by approximately three- and eight-fold, respectively, compared with wild-type dHGF. Similarly, the ability of the mutants to stimulate DNA synthesis in NFS-60 was lower than that of dHGF in terms of EC_{50} (data not shown). As shown in Fig. 4B, the two mutants were more potent than dHGF in the stimulation

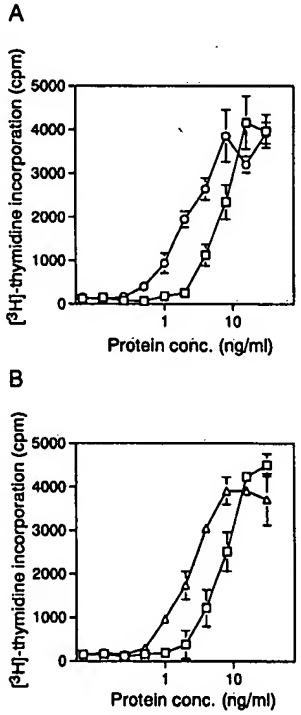

Fig. 3. Effect of dHGF mutants, #2 or #27, on the stimulation of DNA synthesis in rat hepatocytes. Cells were incubated with various concentrations of dHGF, mutants #2 (A) or #27 (B). A: dHGF (□) and mutant #2 (○). B: dHGF (□) and mutant #27 (△). Each value represents the mean ± S.D. of triplicate experiments.

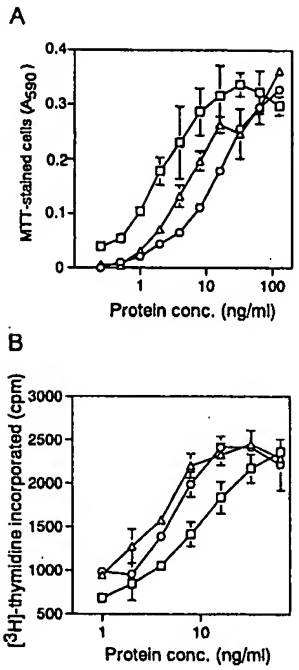

Fig. 4. Mitogenic activity of dHGF mutants, #2 and #27, on (A) NFS-60 (murine myeloblastic cells) or (B) OK (American opossum kidney cells). Cells were incubated with various concentrations of dHGF (□), mutant #2 (○) or mutant #27 (△). Each value represents the mean ± S.D. of the triplicate experiments.

of DNA synthesis in OK cells. In the tested range of 1–64 ng/ml, mutants #2 and #27 showed decreases in EC_{50} by approximately 1.5- and three-fold, respectively, compared with wild-type dHGF. However, the maximum response levels of the two mutants on both NFS-60 and OK cells were comparable to those of dHGF.

3.4. Amino-terminal sequences of mutants #2 and #27

Four positively charged residues are replaced with alanine residues in mutants #2 and #27. These amino acid substitutions may cause a conformational change that can eventually expose a possible protease-sensitive site. Mutant #2 has a predicted amino acid sequence of QAAAA in its N-terminus, which is a possible substrate for the signal peptidase. To examine whether any protease digestion had occurred in the cell or in the conditioned medium, the N-terminal amino acid sequences of the two mutants were determined. The amino-termini of the α chains of both mutants were blocked, as is the case with dHGF. The amino acid residues from the second to the fifth positions of #2 were AAAA, and those of #27 were RKRR. The amino-terminal sequences of the β chains of both mutants were VVNGI. These results indicate that the substitutions of amino acid residues did not induce the susceptibility to protease.

3.5. Affinity of mutants #2 and #27 to heparin

Conditioned medium of cells expressing #2 or #27 was loaded on a HiTrap heparin column and the bound protein was eluted with NaCl-containing buffer. As shown in Fig. 5, the mutants #2 and #27 were eluted from the column at NaCl concentrations of 0.78 M and 0.82 M, respectively, whereas dHGF was eluted at that of 1.14 M.

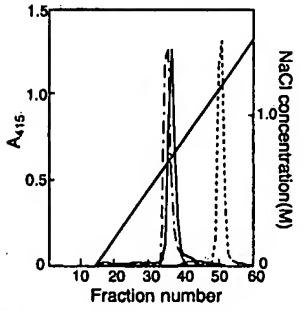

Fig. 5. HiTrap heparin column chromatography of dHGF and dHGF mutants #2 and #27. Conditioned medium of a cell line expressing dHGF (dotted line), dHGF mutants #2 (dot-dashed line) or #27 (solid line) was loaded on a HiTrap heparin column as described in Section 2.

4. Discussion

It has been reported that dHGF is distinguishable from HGF in several ways. First, dHGF is more potent than HGF in the stimulation of DNA synthesis in cultured adult rat hepatocytes [10,22] and in epithelial cells [23]. Conversely, HGF is more potent in the stimulation of DNA synthesis in mesenchymal cells [23]. Second, dHGF is over 70-fold less soluble than HGF in PBS [23]. Finally, several monoclonal antibodies raised against dHGF do not recognize HGF or 2-mercaptoethanol-reduced dHGF [23], demonstrating that the two forms of HGF are different in tertiary structure. In the present study, to obtain further insight into the structure-function relationships of HGF and dHGF, we analyzed dHGF by alanine scanning mutagenesis.

Our analysis of mutants with alanine substitution(s) in the N-terminal domain of dHGF has identified two sites important for its biological activity. One of the sites, 59DKARK, is in the hairpin loop structure. This suggests that the hairpin loop structure of dHGF is a structurally or functionally important region, as is the case with HGF [22,34]. However, the finding that the five other point mutations in the hairpin loop structure, D37A, R42A, R45A, K47A and K54A, did not affect the biological activity (Fig. 2A) indicates that the integrity of the structure is not crucial for the mitogenic activity. The other mutant, ENKD89ANAA, which has no detectable mitogenic activity on rat hepatocytes, has mutations in the region between the hairpin loop structure and the first kringle domain. Unexpectedly, none of the single point mutations in 59DKARK and 89ENKD abolished the biological activity (Fig. 2B), suggesting that no specific residue in the two stretches is directly involved in the interaction with the receptor. Lokker et al. demonstrated that the production levels of HGF (not dHGF) mutants with mutations at the positions corresponding to 59DKARK and 89ENKD (termed D90A,K91A,R93A,K94A and E121A,N122A,K123A,D124A, respectively in [28]) are low in human kidney 293 cells [28]. Taken together, these results suggest that the two regions may be important for the folding of both HGF and dHGF into proper structures.

A comparison of the results obtained from the mutational study of dHGF (present study) and that of HGF [28] clarifies the similarities of and the differences between the two forms of HGF. An HGF mutant, K52A,D54A, had lower mitogenic activity than did wild-type HGF on hepatocytes [28], while the

corresponding dHGF mutant (KID21AIA) had a specific activity comparable to that of wild-type dHGF (Fig. 2A). This suggests that the role of the KID sequence in the biological activity is different between HGF and dHGF. HGF mutants, H114A,E115A,D117A and D171A, could not stimulate DNA synthesis in rat hepatocytes, due to their inability to bind to the receptor [28], whereas the corresponding dHGF mutants (HEND83AANA and RGKD132AGAA, respectively) maintained the biological activity (Fig. 2A). These results strongly suggest that the residues involved in the binding to the receptor are different between dHGF and HGF. The mutational analysis in the first kringle domain also identified two sites, HEH127 and EVRYE159, as sequences essential for the biological function of dHGF. Our analysis of the mutants with a single point mutation in these regions revealed that 128E and 161R are crucial for the biological function of dHGF. Similarly, the alanine substitution of 128E and 166R of HGF, residues that correspond to 128E and 161R of dHGF, respectively, resulted in a loss of receptor binding capability [28]. Therefore, these may be the key residues for both HGF and dHGF to exert the mitogenic activity on rat hepatocytes.

Two mutants (termed #2 and #27) had increased mitogenic activity on rat hepatocytes and OK cells. Replacement of four basic amino acid residues in the N-terminal region with alanine residues resulted in a marked decrease in affinity for heparin (Fig. 5). All of the HGF mutants with no affinity for heparin lose the biological activity [24,34], suggesting that binding to heparin is crucial for the biological function of HGF. Naka et al. showed that cell surface heparin-like molecules are important in the formation of the high-affinity binding sites for HGF [35]. The functional importance of binding to heparin has been reported for other cytokines [36]. However, our analysis of mutants #2 and #27 demonstrated that an about 30% reduction in the heparin binding capability does not diminish the biological activity of dHGF. Further analysis of these two mutants will shed light on the relationship between the heparin binding capability and the biological activity of dHGF.

In some cytokines, mutations in the N-terminal region result in an increase in their biological activity. These include human granulocyte colony-stimulating factor (G-CSF) [37–39], mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) [40] and human tumor necrosis factor-α (TNF-α) [41]. These examples demonstrated that an alteration of the N-terminal region can increase the structural stability or affinity for their signaling receptors. Similar to these cytokines, the mitogenic activity of dHGF on rat hepatocytes was increased when four amino acid residues in the N-terminal region were replaced with alanine residues. Whether or not the mutations influence the protein stability and/or affinity for the receptor is currently under study. It is also of great importance to examine the possibility that the reduction in heparin binding capability is related to the increase in mitogenic activity of the two mutants on rat hepatocytes and OK cells (but not NFS-60).

A previous study has shown that dHGF is not as potent as HGF in stimulating DNA synthesis in mesenchymal cells including NFS-60. However, dHGF is more potent than HGF in stimulating DNA synthesis in epithelial cells including OK cells [23]. Such features of dHGF seemed to be maintained or rather enhanced in mutants #2 and #27. The two mutants may therefore have some advantage in the treatment of diseases that involve epithelial cells in organs such as liver and kidney. Indeed, preliminary in vivo experiments showed that these two mutants are more potent than dHGF in the stimulation of liver function as judged from protein synthesis (M. Kinosaki et al., in preparation).

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EXHIBIT D

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Novel hepatocyte growth factor/scatter factor isoform transcripts in the macaque endometrium and placenta

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Hepatocyte growth factor/scatter factor (HGF/SF) induces proliferation, motility and morphogenesis of cells that express the proto-oncogene for the tyrosine kinase receptor, c-Met. Because these cellular events occur in the endometrium during the menstrual cycle and in placenta during development, we have initiated studies of this growth factor in these tissues from macaques. Several HGF/SF alternatively spliced transcripts have been previously reported in other tissues. However, expression of HGF/SF isoforms in the endometrium has not been studied. Here we describe the relative transcript amounts of HGF/SF isoforms in the endometrium and placenta using RNase protection analyses. During these analyses, we discovered two unexpected protected bands that were found through sequence analyses to represent isoforms similar to the previously reported *NK1* and *NK2* except that they encode a five amino acid deletion in the first kringle domain. We designated these two isoforms as *dNK1* and *dNK2*. Endometrium expressed all of the isoforms; however, *dNK2* was consistently expressed at higher levels than *NK2* transcripts. In contrast, placenta expressed NK2 and *dNK2* mRNA at equal levels, and both *NK1* and *dNK1* were undetectable in placenta. HGF/SF function in endometrium and placenta may involve complex interactions between the isoforms of HGF/SF and those of c-Met.

Key words: endometrium/estrogen/hepatocyte growth factor (scatter factor)/placenta/progesterone

Introduction

Hepatocyte growth factor, also known as scatter factor (HGF/SF), binds heparin and acts in a pleiotropic manner upon cells that express the c-Met proto-oncogenic receptor (Kitamura *et al.*, 1993). These pleiotropic events include cell migration, mitosis, morphogenesis (Sugawara *et al.*, 1997), angiogenesis (Rosen and Goldberg, 1997) and epithelial-to-mesenchymal transition (Fafeur *et al.*, 1997; Fournier *et al.*, 2000). Because these events occur during tissue remodelling and development, it has been proposed that HGF/SF plays a role in regeneration of the endometrium and in development of the placenta. *HGF/SF* mRNA has been localized to endometrial stromal cells and to chorioallantoic mesenchyme of the early conceptus in sheep (Chen *et al.*, 2000). HGF/SF and c-Met transcripts have also been localized to the villous core and cytotrophoblast and decidual glands respectively in human placenta (Clark *et al.*, 1996). Studies with HGF/SF knockout mice show that placental development requires HGF/SF expression (Uehara *et al.*, 1995). Sugawara *et al.* have shown that HGF/SF promotes migration and tubule formation of isolated endometrial epithelial cells *in vitro* (Sugawara *et al.*, 1997). In addition, it has been shown that HGF promotes migration of human endometrial epithelial carcinoma cell lines HEC-1A and KLE *in vitro* (Bae-Jump *et al.*, 1999). However, the actual expression of HGF/SF in primate endometrium has not been shown (Sugawara *et al.*, 1997).

These in-vitro experiments used the full length activated form of HGF/SF. However, there are four known alternatively spliced variants of *HGF/SF* transcripts. First, there is the full length transcript that encodes four plasminogen-like kringle domains, plus a serine protease-like domain. The secreted proforms of HGF/SF must be cleaved to be active, a process that is thought to occur during tissue damage (Nakamura *et al.*, 1989; Silvagno *et al.*, 1995). Second, there is the HGF/SF mRNA that is similar to the full length form except for a 15 bp deletion in the first kringle domain. This form is generally referred to as dHGF (Seki *et al.*, 1990; Shiota *et al.*, 2000). *In vitro*, dHGF and HGF/SF exhibit different biological properties (Shima *et al.*, 1994). Third, a truncated *HGF/SF* transcript called NK2 with antagonistic properties encodes the amino terminus and first two kringle domains but lacks kringles 3 and 4 as well as the carboxy terminus (Chan *et al.*, 1991). Finally, another truncated isoform known as NK1 encodes only the amino terminus and the first kringle domain; antagonist

Table I. Plasma hormone levels

Sample	E+14dP	E-19hrP	E-2dP	E-3dP	E-4dP	E-5dP	E-6dP	E-8dP
Plasma E (pg/ml)	216	154	88	90	346	110	94	115
Plasma P (ng/ml)	8.35	0.5	0.17	0.16	0.54	0.47	0.12	0.39

E = estrogen; P = progesterone; d = day.

as well as agonist effects have been attributed to this isoform (Lokker *et al.*, 1993; Cioce *et al.*, 1996; Jakubczak *et al.*, 1998). The distinct functions of these alternative-spliced HGF/SF isoforms at physiological concentrations are unknown.

To provide further insights into the role of HGF/SF expression in the endometrium and placenta, we examined the relative amounts of the various isoforms in these tissues using the highly sensitive method of RNase protection. Additionally, we analysed the various HGF/SF isoforms in the endometrium as compared to that in placenta. Of interest, we found that the endometrium expresses two novel HGF/SF isoforms, only one of which is expressed by the placenta.

Materials and methods

Animals and treatments

Animal care during these studies was provided by the veterinary staff of the Oregon Regional Primate Research Center (ORPRC) Division of Animal Resources in accordance with the NIH Guide for Care and Use of Laboratory Animals. Eleven pigtail macaques (*Macaca nemestrina*) were ovariectomized and treated sequentially with estradiol (E_2) and progesterone to create artificial menstrual cycles. To create these cycles, a 3 cm Silastic capsule (0.34 cm inner diameter; 0.64 cm outer diameter; Dow Corning, Midland, MI, USA) packed with crystalline E_2 (Steraloids Inc., Wilton, NH, USA) was inserted s.c. at the time of ovariectomy to stimulate an artificial proliferative phase. After 14 days of E_2, a 6 cm Silastic capsule containing crystalline progesterone (Steraloids Inc.) was inserted s.c. for 14 days to stimulate an artificial secretory phase. Removal of the progesterone implant (leaving the E_2 implant in place) completed each cycle and induced two to three days of menstruation. The uteri were collected by mid-ventral laparotomy. Serum levels of E_2 and progesterone in these animals were analysed by radioimmunoassay (see Table I) to confirm retention and function of the implants.

For pigtail macaques, uteri were collected on days 1 (19 h after progesterone withdrawal), 2, 3, 4, 5, 6 and 8 after progesterone implant withdrawal (n = 1 each).

For rhesus macaques, uteri were collected from three treatment groups. Group 1 received implants of E_2 alone for 14 days (n = 2); group 2 received implants of E_2 for 14 days, then E_2 plus implants of progesterone for 14 days (n = 2); and group 3 received E_2 plus progesterone for 14 days then the progesterone implant was removed for 8 days (n = 1).

All placentae were obtained from rhesus macaques undergoing either Caesarean sections or natural births on the days of gestation indicated in Figure 3A and B.

Riboprobe preparation

[^{32}P]UTP-labelled RNA probes were prepared for RNase protection analysis. Using T7 and SP6 RNA polymerases, the 606 bp HGF monkey-specific riboprobe corresponding to nucleotides 395–1000 of the human HGF cDNA sequence (accession no. E03331) was synthesized. The riboprobe used for normalization was that for the monkey-specific S10 ribosomal protein cDNA which corresponds to the human version (nucleotides 171–290, accession no. U14972). The riboprobe corresponding to the 3′ untranslated region of the human NK1 cDNA, nucleotides 1108–1294 (accession no. U46010) (Rubin *et al.*, 1991), was synthesized from a monkey-specific template. All templates were cloned in our laboratory or at the Molecular Core Facility at the Oregon Regional Primate Research Center. All monkey-specific constructs were confirmed by sequencing. An RNA ladder template (Century; Ambion, Inc.) was used to generate RNA-specific size markers. We used the in-vitro transcription protocol as described in Current Protocols in Molecular Biology (Ausubel *et al.*, 1998). For RNase protection, full length probes were purified by electrophoresing on a 6% polyacrylamide denaturing gel. Gel slices containing each probe were mashed in 100 µl of diethylpyrocarbonate (DEPC)-treated water and the probe was eluted by two incubations in 600 µl of 1× proteinase K (PK) buffer (0.3 mol/l NaCl, 0.5% SDS, 10 mmol/l Tris pH 7.5, 200 µg/ml PK, and 20 µg/ml tRNA) for 5–15 min at 37°C. The suspended probe from both incubations was filtered (0.45 µm; Acrodisc), then chloroform/phenol extracted and ethanol precipitated.

RNase protection analyses

Total RNA from tissues was prepared as previously described either by guanidinium isothiocyanate lysis and centrifugation over a caesium chloride cushion or by a single-step acid guanidinium thiocyanate–phenol–chloroform extraction and quantified by measuring optical density. RNase protection analyses were performed as previously described (Ausubel *et al.*, 1998) with minor modifications. Briefly, RNA samples, and tRNA as a negative control, were precipitated with excess amounts of the appropriate gel-purified [^{32}P]UTP-labelled probes. The pellet was resuspended in 30 µl of annealing buffer [40 mmol/l PIPES (pH 6.4), 0.4 mol/l NaCl, 1 mmol/l EDTA, 80% formamide] and allowed to hybridize overnight at 42°C. Unhybridized RNA was digested with RNase A (50 µg/ml) and RNase T1 (4 µg/ml) for 30 min at 37°C. RNases were then removed by treatment with proteinase K and extraction with phenol/chloroform/isoamyl alcohol. After ethanol precipitation, the RNA pellet was resuspended in 90% formamide loading buffer, denatured at 85°C for 10 min and electrophoresed on a 6% polyacrylamide denaturing gel. The dried gel was exposed to film at –70°C for the time periods indicated in the figure legend.

Densitometry

Relative levels of HGF isoform expression were derived as compared to the expression level of ribosomal protein RNA, S10. Densitometry was performed using the Bio-Rad Molecular Analyst Software program and Densitometer 700 Scanner. The optical density of each band was obtained and background for that lane was subtracted. Graphs were derived using the Microsoft Excel program.

RT–PCR

Reverse transcription was performed with total RNA from macaque endometrium in the proliferative phase. Briefly, 2 µg of total RNA in 11 µl DEPC-treated H_2O was heated to 70°C for 10 min and

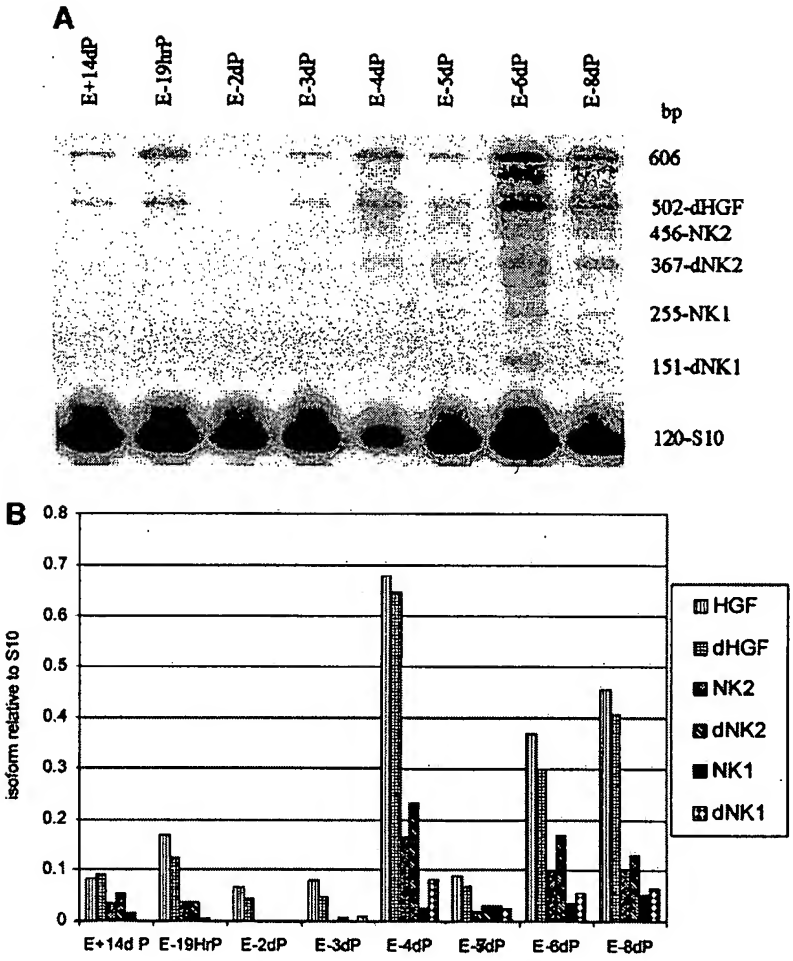


Figure 1. RNase protection using the 606 bp *HGF* monkey-specific riboprobe (A) and densitometry analyses (B) of total endometrial RNA isolated from pigtail macaques treated with estrogen alone (E) or estrogen and progesterone (E+P) as indicated. The uterus was removed during continuous estradiol treatment at the indicated times after progesterone withdrawal, for example, E-19hrP (19 h after progesterone withdrawal), E-3dP (3 days after progesterone withdrawal) etc. Levels of RNA loaded were normalized to S10 ribosomal protein RNA. The protected band size for each isoform is listed on the right in (A). These sizes were determined by the RNA ladder (not shown) as stated in Materials and methods. Exposure to film was for 24 h.

placed on ice to maintain the denatured state. The following was added: 1× 1st strand buffer (Clontech, Palo Alto, CA, USA), 10 mmol/l dithiothreitol, 2 mmol/l dNTP, 20 IU RNasin, 0.5 µmol/l penultimate 3′ primer and incubated for 1 h at 56°C for *NK1*-specific 3′ untranslated region (UTR) primer and at 53°C for *NK2*-specific 3′ *UTR* primer. *NK1* and *NK2* transcripts each possess unique 3′ UT regions (Chan *et al.*, 1991; Cioce *et al.*, 1996). After incubation, 100 IU of reverse transcriptase (MMLV; Promega, Madison, WI, USA) was added and the mixture was incubated for 45 min at 42°C with inactivation at 65°C for 10 min.

PCR for 35 cycles was performed with the Advantage® cDNA PCR kit by Clontech. Briefly, 1× cDNA PCR reaction buffer, ~1.0 ng cDNA template from RT mixture, 0.2 mmol/l dNTP mix, 0.2 µmol/l 5′ primer, 0.2 µmol/l 3′ nested primer, 1× Advantage® polymerase mix were combined, denatured at 94°C for 30 s, and cycled under the following conditions: 94°C for 40 s, annealed at 56°C for 1 min, and 68°C for 2 min. Products were electrophoresed on a 2% gel containing 0.5 µg/ml ethidium bromide. The very bottom of each expected size band, ~365 bp for *NK1* and ~534 bp for *NK2*, was excised and reamplified using the same PCR protocol and primers. The resulting products were then subcloned into the pCRII vector (Invitrogen) and sequenced.

We used the following primers for *NK1* from accession no. u46010 (Rubin *et al.*, 1991). 5′ primer: 5′-ACTGCATCATTGGTAAAGGAC-3′ (nucleotides 434–454); 3′ penultimate primer: 5′-CTTGTCAGCC-ATTCAGTTTTCC-3′ (nucleotides 1372–1393); 3′ nested primer: 5′-TGCATTTGCACGAACAAC-3′ (nucleotides 782–799).

We used the following primers for *NK2* from accession no. m77227 (Chan *et al.*, 1991): 5′ primer: 5′-GCATCATTGGTAAAGGAC-3′ (nucleotides 465–482); 3′ penultimate primer: 5′-TCAGAAAAGCT-GGGTAAG-3′ (nucleotides 1161–1178); 3′ nested primer: 5′-TGTC-ACTCACCAGAAGAAG-3′ (nucleotides 981–999).

Results

Endometrial HGF/SF isoform expression

We used RNase protection analyses to determine the relative expression of the different HGF/SF isoforms. Endometrial samples from eight different animals were analysed three times by this assay. As can be seen in the representative analysis in Figure 1A, the lowest level of endometrial *HGF/SF* isoform expression was evident at the end of the artificial luteal phase,

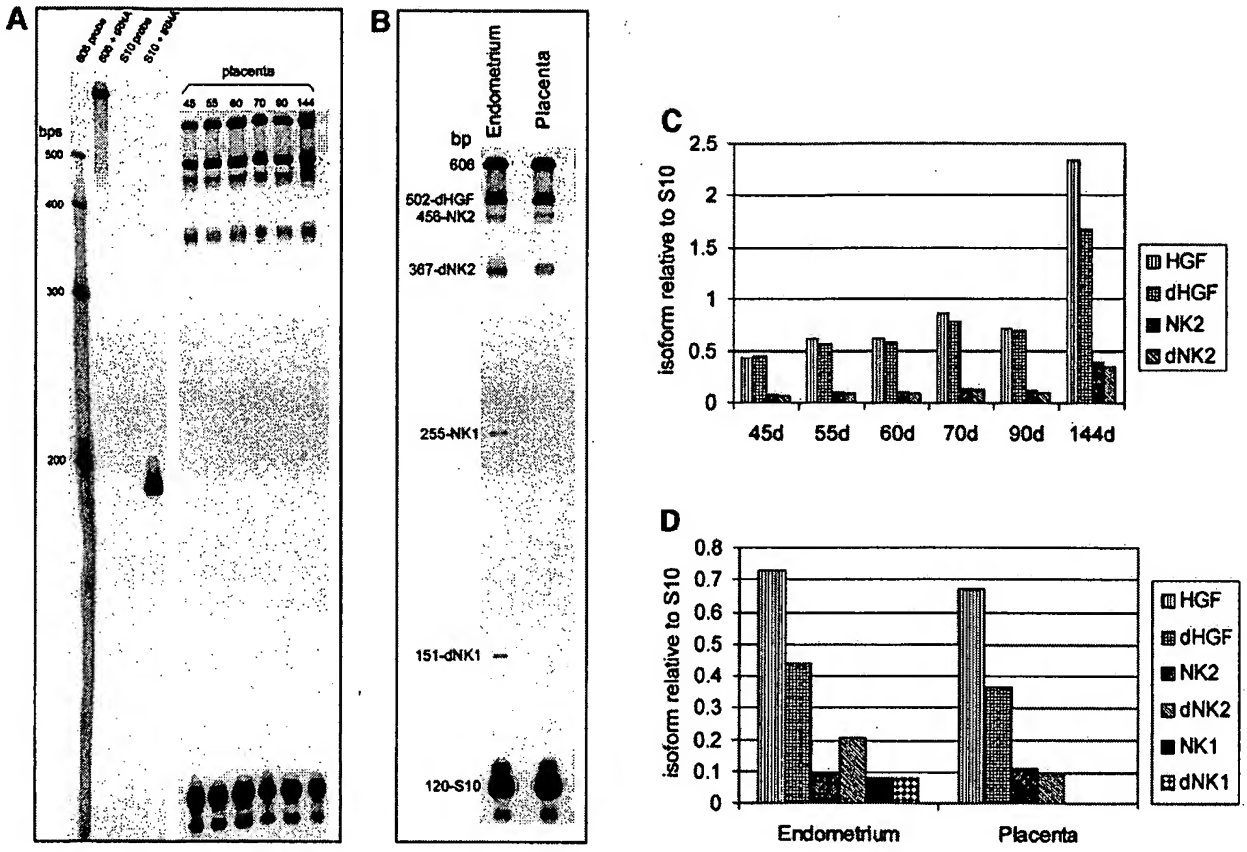


Figure 2. (**A**) RNase protection of total placental RNA isolated from days 45–144 of gestation as indicated. This analysis shows the ladder used for all RNase protection analyses as well as the controls, probe alone and probe with tRNA, to determine if background binding with tRNA was present. No background was detected. Exposure to film was for 20 h. (**B**) Endometrial sample from E-8dP (estrogen alone for 8 days), and placental sample from 157 days of gestation, both from rhesus macaques. Levels of RNA loading were equal for all samples in **A** and **B**. Exposure to film was for 3 days. (**C**) Graph of densitometry results from Figure 3A. (**D**) Graph of densitometry results from Figure 3B.

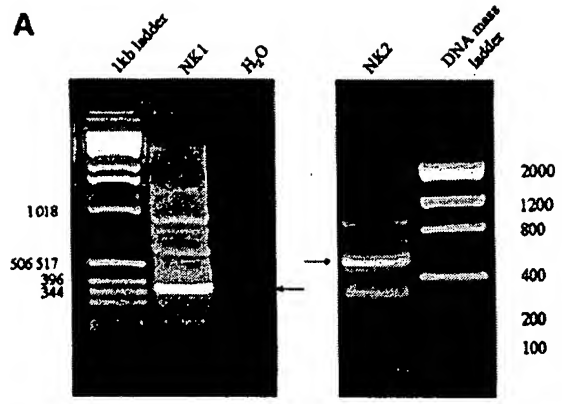
when progesterone levels were high, and the highest levels were found in estrogen-treated animals after progesterone was withdrawn. Serum hormone levels of the macaques used in this RNase protection analysis are shown in Table I. We detected the expected sized protected bands for full length *HGF/SF* (~606 bp), *dHGF* (~502 bp), *NK2* (~456 bp) and *NK1* (255 bp). Both *HGF/SF* and *dHGF* were expressed in equal amounts and were generally expressed at 3- and 7-fold higher levels than the *NK2* and *NK1* isoforms respectively (Figure 1B). In addition, two unexpected protected bands (367 and 151 bp) were observed. These same protected bands were also seen in estrogen + progesterone, E-8dP and E-14dP rhesus macaque endometrial RNA samples (data not shown). We named these two unexpected bands *dNK2* (367 bp) and *dNK1* (151 bp) because we hypothesized they had deletions in the first kringle domain of NK2 and NK1 respectively. The resulting band representing *dNK1* or *dNK2* in our assays was always the larger of the two protected fragments on either side of the 15 bp deletion. In other words, the region 5′ of the 15 bp deletion would run at the bottom or off the gel because of its small size (86 bp). These results show that *dNK2* was consistently expressed at higher levels than the previously reported *NK2* (Figure 1B). When detectable, levels of the various isoforms relative to full length *HGF* transcripts did not appear to change with hormonal treatment.

Placental HGF/SF isoform expression

In all six placentae from different animals, levels of full length *HGF/SF* and *dHGF* mRNA were essentially equal as seen in the endometrium. However, in the placenta, the truncated isoforms tended to be even lower than in endometrium or undetectable. *NK2* and *dNK2* were expressed at similar levels but *NK1* and *dNK1* transcripts were not detected (Figure 2A). In the RNase protection analyses comparing endometrium with placenta (Figure 2B), *NK1* and *dNK1* transcripts were not detectably expressed in placenta, whereas *NK2* was expressed at comparable levels and *dNK2* at lower levels to those detected in endometrium (Figure 2C and D). Consistently, an unknown isoform band at ~520 bp in endometrium was absent in placental RNA samples. This protected 520 bp band is not described in this report. However, we did evaluate the unexpected *dNK1* and *dNK2* bands (see below).

Isolation and sequencing of two novel isoforms

We performed RT–PCR as described in Materials and methods using RNA isolated from estrogenized endometrium. Figure




```
B   2 CAAGGGAACAGTATCTATCACTAAGAGTGGCATCAAATGTCAGCCCTGG 51      dNK1
    462 CAAGGGAACAGTATCTATCACTAAGAGTGGCATCAAATGTCAGCCCTGG 510   U46010

    52 AGTTCCATGATACCACACGAACAC.....AGCTATCGGGG 86
    511 AGTTCCATGATACCACACGAACACAGCTTTTTGCCTTCGAGCTATCGGGG 560

    87 TAAAGACCTACAGGAAAACTACTGTCGAAATCCTCGAGGGGAAGAAGGGG 136
    561 TAAAGACCTACAGGAAAACTACTGTCGAAATCCTCGAGGGGAAGAAGGGG 610

    137 GACCCTGGTGTTTCACAAGCAATCCAGAGGTACGCTACGAAGTCTGTGAC 185
    611 GACCCTGGTGTTTCACAAGCAATCCAGAGGTACGCTACGAAGTCTGTGAC 660

    186 ATTCCTCAGTGTTCAGAAGGTAAATAAACCTGAATGCCATGTGGGCCATT 235
    661 ATTCCTCAGTGTTCAGAAGGTAAATAAACCTGAATGCCATGTGGGCCATT 710

    236 CTATTCCCCCTATGTGTAGAACTGTAACTCACATTAAAGGTTAACAGCAA 285
    711 CTATTCCCCCTATGTGTAGAACTGTAACTCACATTAAAGGTTAACAGCAA 760

    286 CGAA 289
    761 CGAA 764

C   2 AAGGGACGCAGCTACAAGGGAACAGTATCTATCACTAAGAGTGGCATCAA 52      dNK2
    476 AAAGGACGCAGCTACAAGGGAACAGTATCTATCACTAAGAGTGGCATCAA 525   M77227

    53 ATGTCAGCCCTGGAGTTCCATGATACCACTCGAACAC..... 89
    526 ATGTCAGCCCTGGAGTTCCATGATACCACACGAACACAGCTTTTTGCCT 574

    90 ...AGCTATCGGGGTAAAGACCTACAGGAAAACTACTGTCGAGATCCCC 135
    575 TCGAGCTATCGGGGTAAAGACCTACAGGAAAACTACTGTCGAAATCCTC 623

    136 GAGGGGAAGAAGGGGGACCCTGGTGTTTCACAAGCAATCCAGAGGTAC 184
    624 GAGGGGAAGAAGGGGGACCCTGGTGTTTCACAAGCAATCCAGAGGTAC 671

    185 GCTACGAAGTCTGTGACATTCCTCAGTGTTCAGAAGTTGAATGCATGACC 235
    672 GCTACGAAGTCTGTGACATTCCTCAGTGTTCAGAAGTTGAATGCATGACC 721

    236 TGCAATGGGGAGAGTTATCGAGGTCTCATGGATCATACAGAATCAGGCA 285
    722 TGCAATGGGGAGAGTTATCGAGGTCTCATGGATCATACAGAATCAGGCA 770

    286 AGATTTGTCAGCGCTGGGATCATCAGACACCACACCGGCACAAATTCTTG 336
    771 AGATTTGTCAGCGCTGGGATCATCAGACACCACACCGGCACAAATTCTTG 820
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Figure 3. (A) RT–PCR amplification of RNA from E-8dP rhesus macaque endometrium. Arrows indicate the expected size bands for NK1 (365 bp), left panel and NK2 (534 bp), right panel.
(B) Representative Bestfit analysis of dNK1 sequence determined after excision of band from left panel of gel in A, subcloning, and sequence comparison with NK1 (accession no. U46010).
(C) Representative Bestfit analysis of dNK2 sequence determined after excision of band from right panel of gel in A, subcloning, and sequence comparison with NK2 (accession no. M77227).

3A shows the products from *NK1*- or *NK2*-specific primers. Fortunately, these two isoforms have distinct 3′ untranslated regions to which we could design isoform-specific primers. Because it is difficult to isolate such closely related bands (of 15 bp difference) by electrophoresis, we excised the lower half of the major bands that were the predicted size (arrows) for *NK1* and *NK2* bands and subcloned each into the pCRII vector. The spurious bands were probably due to the primers binding non-specifically. However, this was the annealing temperature at which we received adequate specific product to isolate from the gel, purify, and subclone.

After subcloning and plating, we isolated five clones from each plate, prepared DNA, and had sequencing performed. We took the resulting sequences and compared them to the respective *NK1* or *NK2* sequence already in the database. We used the program Bestfit in GCG; representative comparisons are seen in Figure 3B and C. At least three out of the five clones for each isoform contained the same 15 bp deletion.

Discussion

This paper reports the discovery of two new truncated *HGF/SF* isoforms that encode a deletion in the first kringle domain and are truncated after either the first kringle (dNK1) or the second kringle (dNK2). In previous reports on truncated forms, RNase protection analyses were not used and therefore the different bands were not evident from Northern analyses. The 15 bp deletion found in dNK1 and dNK2 is the same deletion found in dHGF. Estrogenized endometrium consistently expressed *dNK2* at higher levels than *NK2* mRNA, whereas *NK1* and *dNK1* levels were approximately equal in their degree of expression. In contrast, the placenta, which did not express either *NK1* or *dNK1* at detectable levels, expressed *NK2* and *dNK2* in approximately equal amounts.

In previous reports on deletions in the first kringle, the functions of deleted forms are unclear. One function attributed to this deletion sequence is heparin binding. Another family of heparin binding proteins is the fibroblast growth factor (FGF) family. The amino acid sequence deleted in the first kringle of dNK1 and dNK2, FLPSS, is one of the conserved sequences in all seven members of the FGF family, and its deletion in basic FGF modulates heparin-binding activity (Seno *et al.*, 1990). Also, both the hairpin loop and the first kringle domain have been shown to play a role in heparin binding by HGF/SF. HGF/SF and NK1 require heparin sulphate glycosaminoglycan (HSGAG) to bind c-Met efficiently as seen in studies of HSGAG-deficient CHO cells. Shima *et al.* have reported that dHGF elutes slightly earlier than HGF/SF on heparin-affinity high performance chromatography (Shima *et al.*, 1991), suggesting that the 15 bp deletion does change the heparin binding property. Heparin has been localized to basement membrane surrounding glands and blood vessels in the endometrium (Aplin *et al.*, 1988). We have localized c-Met mRNA and protein to glandular epithelium and to blood vessels in primate endometrium (data not shown), while in ovine endometrium c-Met mRNA has been localized exclusively to luminal and glandular epithelium (Chen *et al.*, 2000). As a result, the deletion in the first kringle domain may affect

the localization of these isoforms to heparinized regions and their proximity to the c-Met receptor in the endometrium. In addition, HGF/SF, both in its active and precursor forms, as well as NK1 and NK2, bind to the extracellular matrix components, thrombospondin-1 and fibronectin (Lamszus *et al.*, 1996), though this study did not examine the effects of the deletion in the first kringle and its effects on binding to these proteins. Kringle structures have also been shown to facilitate protein–protein interactions (Patthy *et al.*, 1984). Therefore, the conformational changes caused by the lack of five amino acids in the first kringle probably affects the binding of HGF/SF isoforms to several molecules in the extracellular matrix, including heparin as well as unknown proteins.

The functions of NK1 and NK2 have been elusive. NK2 was the first described truncated HGF/SF isoform. Unlike HGF/SF, NK2 does not induce DNA synthesis in B5/589 human mammary epithelial cells. However, a 10–20-fold molar excess of NK2 over HGF/SF can inhibit DNA synthesis by 50% (Chen *et al.*, 1991). Therefore, NK2 could act as an antagonist for HGF/SF-induced DNA synthesis. For this DNA synthesis assay (Chan *et al.*, 1991), NK2 was isolated from SK-LMS-1 mammalian cells that normally produce this protein and then purified over a heparin–Sepharose column. It is conceivable that dNK2 does not elute from this column at the same time as NK2 due to a possible change in affinity for heparin. Therefore, it is not clear whether this assay used NK2 or dNK2 or both. Conditioned media from a naturally NK2-producing cell line, SBC-5, causes cell scattering on SBC-1 and SBC-2 cell lines. However, a sensitive method of HGF/SF detection was not used in this protocol (Itakura *et al.*, 1994). Therefore, this cell scattering could have been due to contamination with HGF/SF. Transfected cDNA for NK2 does not cause angiogenesis *in vivo* when compared to HGF/SF (Silvagno *et al.*, 1995). However, NK2 causes c-Met phosphorylation and can induce cell dissociation of, but not mitosis in, MDCK cells, although it has a 30-fold lower specific activity than HGF/SF (Hartmann *et al.*, 1992). Therefore, it appears that NK2 activates the c-Met receptor and stimulates breakdown of cell–cell contacts in epithelial cells in culture. Whether or not dNK2 also binds to heparin, c-Met, or acts as a stimulator of cell scattering remains to be determined.

NK1 has been isolated and analysed by several laboratories. At a 50-fold molar increase over HGF/SF, NK1 phosphorylates the c-Met receptor and acts as a competitive HGF/SF antagonist but lacks intrinsic mitogenic activity in primary rat hepatocyte cultures (Lokker and Godowski, 1993). At an 80-fold molar increase over HGF/SF, NK1 stimulates DNA synthesis (Cioce *et al.*, 1996). Overexpression of NK1 in transgenic mice has been shown to have similar yet reduced effects to overexpression of HGF/SF in transgenic mice. These effects include liver hyperplasia, kidney epithelial hyperplasia, aberrant striated muscle formation and increased tumorigenesis in older mice. Increased NK1 expression may facilitate oligomerization with HGF/SF and c-Met dimerization and activation. However, NK1 was overexpressed in many of these transgenic mouse tissues at levels equal to HGF/SF (Jakubczak *et al.*, 1998) which are not physiological levels. As a result, it is unclear how NK1 functions *in vivo*.

We have localized HGF/SF in macaque placenta (not shown), consistent with that previously reported for human placenta (Clark *et al.*, 1996). It has been proposed that HGF/SF expressed in the villous core acts on the trophoblastic cells in a paracrine manner to cause trophoblast growth as well as migration into the decidua (Lail-Trecker *et al.*, 1998). In the placenta, the absence of NK1 and dNK1, which can antagonize HGF/SF action, may favour unopposed HGF/SF action. However, in the endometrium, the presence of NK1 and dNK1 may provide a moderating effect during estrogen-stimulated HGF/SF expression. Northern blot analyses of placental expression of HGF/SF isoforms has been reported previously (Kitamura *et al.*, 1993). However, our work is the first report that *NK1* and *dNK1* mRNA are not detectable in placenta. Also, this is the first report showing that endometrium expresses all previously reported isoforms including the two novel isoforms we detected by RNase protection analysis and confirmed by RT–PCR, cloning and sequencing. This difference in HGF/SF isoform expression may be due in part to a difference in expression of alternative splicing proteins between endometrium and placenta, such as that described for the splicing factor SC35 (Nie *et al.*, 2000).

In conclusion, we have isolated two novel, alternatively spliced, truncated variants of HGF/SF, designated as *dNK1* and *dNK2*. The placenta lacked *NK1* and *dNK1*. Endometrium expressed higher levels of *dNK2* than the previously described *NK2*. Like dHGF, dNK1 and dNK2 may have different solubilities and tertiary structures when compared with NK1 and NK2. Also, since dHGF and HGF/SF require cleavage to be active and the truncated isoforms do not possess this cleavage region, the truncated isoforms may be continually active in both endometrium and placenta. There may be oligomerization of different isoforms and complex binding patterns to the c-Met isoforms which play important roles in estrogen-dependent HGF signalling. Whether the endometrial signalling pathways induced by these truncated isoforms differ from that of the full length forms, and whether they play important roles in endometrial and placental physiology, are matters for future research.

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EXHIBIT E

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Affinity evaluation of gelatin for hepatocyte growth factor of different types to design the release carrier

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Abstract—The objective of this study was to investigate the physicochemical interaction of hepatocyte growth factor (HGF) and its variant with 5 amino-acid residues deleted (dHGF) with an acidic gelatin for the design of factors release from the gelatin hydrogel. When the interaction of HGF or dHGF with gelatin-immobilized agarose beads was evaluated by Scatchard binding assay, the dissociation constant of dHGF was higher than that of HGF, although the two proteins had a similar binding ratio. dHGF was released more rapidly from the hydrogel of acidic gelatin than HGF. *In vivo* release study with ^{125}I-labeled HGF or dHGF in mice subcutis showed that HGF was released from the gelatin hydrogel as a result of hydrogel degradation. In contrast, dHGF was rapidly released by a simple diffusion from the gelatin hydrogel. From electrophoresis experiments, mixing with the acidic gelatin enabled HGF to complex and suppressing the trypsin-digested molecular weight loss, in marked contrast to that of dHGF. In addition, the percentage of HGF recognized by the antibody was reduced by the gelatin complexation, but that of dHGF was not. We conclude that unlike dHGF, HGF has a strong affinity for the acidic gelatin, resulting in the controlled release of HGF accompanied with hydrogel degradation of the release carrier.

Key words: Hepatocyte growth factor; gelatin; release profile; hydrogel.

INTRODUCTION

The biomedical technology and methodology to create a local environment for induction of regeneration of tissues and organs is named tissue engineering. The key factors include cells, a scaffold for their proliferation and differentiation, and growth factors. They are generally combined and used, although the combination manner depends on the type of target tissues and organs to be regenerated. The growth factor is often required to promote tissue regeneration. In addition, considering the usage

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of cells in the body, it is no doubt that a sufficient supply of nutrients and oxygen to the cells transplanted is vital for their survival and functional maintenance [1, 2]. Without sufficient supply, only a small number of cells pre-seeded in the scaffold or migrated into the scaffold from the surrounding tissue would survive. It will be a promising way for the vital supplies to induce new formation of vascular networks by making use of angiogenic growth factors [2].

Hepatocyte growth factor (HGF) is originally discovered as a protein factor to accelerate hepatocyte proliferation [3]. However, it has been recently demonstrated that HGF has diverse potentials for proliferation, differentiation, mitogenesis, motogenesis and morphogenesis of various cells [4–6].

In addition to the cDNA originally reported, another major variant which lacks 15 nucleotides, encoding 5 amino-acid residues (FLPSS sequence) in the first kringle domain (dHGF), has been isolated [4, 7, 8]. However, the significance of dHGF remains to be established. Structure–function studies with partially truncated HGF molecules [9, 10] have demonstrated that three N-terminal domains in the α-chain of HGF (the hairpin and the first two kringles) are essential for the biological function of HGF. This implies that the deletion of 5 amino acids in the first kringle might affect the biological activity of HGF. It has been reported that the structure-function analysis of dHGF by alanine scanning mutagenesis [11] and antigen specificity of monoclonal antibodies [12] indicate a structural difference between HGF and dHGF.

On the other hand, we have prepared a biodegradable hydrogel from the acidic gelatin with an isoelectric point (IEP) of 5.0 which can form a complex with HGF and succeeded in the controlled release of biologically active HGF. As expected, the acidic gelatin hydrogel incorporating HGF was found to enhance its *in vivo* vascularization effect of HGF [13], in marked contrast to HGF in the solution form.

This objective of this study is to obtain fundamental information about the interaction of HGF or dHGF with the acidic gelatin. Sorption experiments based on Scatchard binding analysis were performed to investigate the interaction between HGF or dHGF and the acidic gelatin. The *in vitro* and *in vivo* time profiles of HGF and dHGF release from the hydrogels of acidic gelatin were evaluated while the latter was compared with that of gelatin hydrogels. We examined the change in the molecular properties of HGF or dHGF before and after complexation with the acidic gelatin in terms of the electrophoretic and enzyme-linked immunosorbent assays to evaluate the protein–gelatin interaction.

MATERIALS AND METHODS

Materials

A gelatin sample with an IEP of 5.0 (99 kDa), prepared through an alkaline process of bovine bone collagen, was kindly supplied by Nitta Gelatin (Osaka, Japan) and named 'acidic' gelatin, based on the IEP. Human recombinant HGF (Lot No. GJ09909B) was purchased from PeproTech EC (London,

UK) and human recombinant dHGF was kindly supplied by Research Institute of Life Science, Snow Brand Milk Products (Tochigi, Japan). $Na^{125}I$ aqueous solution in 0.1 M NaOH (NEZ033, 740 MBq/ml) and N-succinimidyl-3-(4-hydroxy-3,5-di-[^{125}I]iodophenyl) propionate ([^{125}I]Bolton–Hunter reagent, NEX-120H, 147 MBq/ml in anhydrous benzene) were purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). N-Hydroxysuccinimidyl (NHS)-immobilized agarose beads were purchased from Amersham Biosciences (Piscataway, NJ, USA). A human HGF ELISA kit (Immunis® EIA) was purchased from Institute of Immunology (Tokyo, Japan). Other chemicals were purchased from Wako (Osaka, Japan) and used without further purification.

Immobilization of acidic gelatin to agarose beads

The suspension of NHS-immobilized agarose beads in ethanol (1.0 ml) was centrifuged at 1×10^4 rpm for 10 min and the supernatant was removed, then the beads were washed with 1 mM cold HCl (2.0 ml) three times. Next, 1.0 ml of gelatin solution (1.0 mg/ml) in a coupling buffer (0.2 M $NHCO_3$ and 0.5 M NaCl aqueous solution) was added to the beads. Next, the agarose beads were washed with 2 ml deactive buffer A (0.5 M ethanolamine and 0.5 M NaCl aqueous solution, pH 8.3) three times and 2 ml deactive buffer B (0.1 M acetic acid and 0.5 M NaCl, pH 4.0) three times. The set of washing procedure was repeated three times, and the acidic gelatin-immobilized agarose beads prepared were stored in 2 ml 0.05 mM phosphate-buffered saline solution (PBS, pH 7.4). The amount of gelatin immobilized was 0.3 mg/mg bead and determined by measuring the protein amount in the reaction mixture supernatant before and after immobilization reaction. Non-immobilized agarose beads (as a control study) were prepared by using the coupling buffer without gelatin.

Sorption assay of HGF or dHGF to acidic gelatin

Preparation of ^{125}I-radiolabeled HGF and dHGF was performed according to the chloramine T method reported previously [14, 15]. The isotherm experiment of HGF or dHGF sorption to the gelatin-immobilized beads was performed. Briefly, aqueous solution containing different amounts of ^{125}I-labeled HGF or dHGF (100 μl) was added to the suspension of gelatin-immobilized agarose beads (100 μl, 45 μg gelatin/μl agarose beads) and the mixture was left for 48 h at 37°C, which was experimentally found to be an condition to achieve the equilibrium sorption of HGF or dHGF, irrespective of the HGF or dHGF concentration. Then, the supernatant was separated from the agarose beads. The equilibrium concentration of free HGF or dHGF in the solution (C_f) was determined by measuring the radioactivity. The molar ratio of HGF or dHGF sorbed to gelatin (r) was calculated based on HGF, dHGF and gelatin molecular masses of 90, 90 and 100 kDa, respectively. The r/C_f was plotted as a function of according to the Scatchard binding model [16]. The dissociation constant (K_d) and the binding molar ratio of HGF or dHGF to the

acidic gelatin were obtained from the slope and the intercept of the r/C_f *vs.* r line at $r = 0$.

Preparation of acidic gelatin hydrogel

A hydrogel was prepared by chemically cross-linking the acidic gelatin with glutaraldehyde. Briefly, 50 ml of 5 wt% acidic gelatin aqueous solution was mixed with glutaraldehyde aqueous solution (25 wt%) to give a final concentration of 5.0 mM. The mixing solution was poured into a plastic mold (8×8 cm^2), followed by leaving it for 24 h at 4°C for gelatin cross-linking. Then, the resulting gelatin hydrogel sheet was immersed in 0.1 M glycine aqueous solution at 37°C for 1 h to block the residual aldehyde groups of glutaraldehyde. The cross-linked hydrogel sheet was cut into square-shaped sheets of approximately 3 mg (3×3 mm^2), and these hydrogel sheets were rinsed three times with double-distilled water (DDW) at 37°C and freeze-dried. The weight of hydrogels before and after swelling in DDW for 24 h at 37°C was measured to calculate the water content which is the weight ratio of water present in the hydrogel to the wet hydrogel. The water content of the hydrogels was 96.0 wt%.

Estimation of in vitro *HGF or dHGF release from acidic gelatin hydrogel*

An aqueous solution of ^{125}I-labeled HGF or dHGF was sorbed into one freeze-dried gelatin hydrogel sheet to prepare a gelatin hydrogels incorporating ^{125}I-labeled growth factor.

The *in vitro* release test of ^{125}I-labled HGF or dHGF from the gelatin hydrogel was carried out on a shaker at 37°C. Gelatin hydrogels incorporating ^{125}I-labeled HGF or dHGF was placed in 1 ml PBS and the buffer was changed periodically. The radioactivity of the buffer was measured on a gamma counter (ARC-301B, Aloka, Tokyo, Japan). The percentage of protein released was calculated from the ratio of cumulative radioactivity of the supernatant to the radioactivity of gelatin hydrogels before placing in PBS.

Estimation of in vivo *degradation of acidic gelatin hydrogel*

The gelatin hydrogels prepared were radioiodinated by use of [^{125}I]Bolton–Hunter reagent [17]. Briefly, 20 μl of [^{125}I]Bolton–Hunter reagent solution in anhydrous benzene was bubbled with dry nitrogen gas until benzene was completely evaporated. Then, 1 ml of PBS was added to the dried reagent to prepare aqueous solution of [^{125}I]Bolton–Hunter reagent. The reagent solution prepared was impregnated into the gelatin hydrogel sheet at a volume of 20 μl of per sheet. The resulting hydrogel sheets were kept at 4°C for 3 h to introduce ^{125}I into the amino groups of gelatin. The radioiodinated gelatin hydrogel sheets were rinsed in DDW, which was exchanged periodically at 4°C for 4 days, to exclude non-coupled, free ^{125}I-labeled reagent from ^{125}I-labeled gelatin hydrogel sheets. When measured periodically the

radioactivity of DDW returned to a back ground level after 3-day rinsing. No shape change of hydrogel sheets was observed during radiolabeling and the subsequent rinsing process, irrespective of the hydrogel type. The ^{125}I-labeled gelatin hydrogels were implanted in the back subcutis of ddY mice (3 mice per group, 6–7 weeks old, Shimizu Laboratory Supply, Kyoto, Japan) under pentobarbital anesthesia. At 1, 3, 7, 10 and 14 days after hydrogel implantation, the radioactivity of hydrogels explanted was measured on a gamma counter. Next, the mouse back skin around the hydrogel implanted site was cut into a strip of 3×5 cm^2 and the corresponding facial site was thoroughly wiped off with a filter paper to absorb ^{125}I-labeled gelatin. The radioactivity of the skin strip and the filter paper was measured to evaluate the remaining radioactivity of tissue around the hydrogel implanted. The ratio of total radioactivity measured to the radioactivity of hydrogel implanted initially was expressed as the percentage of remaining activity for hydrogel degradation. In each experimental group consisted of 15 mice, while 3 mice were killed at each time point for *in vivo* evaluation. The half-life time periods of gelatin hydrogels were evaluated from the time-course curve of radioactivity remaining of ^{125}I-labeled gelatin hydrogel. All the animal experiments were done according to the Institutional Guidance of Kyoto University on Animal Experimentation.

Estimation of in vivo *HGF or dHGF release from acidic gelatin hydrogel*

Gelatin hydrogels incorporating ^{125}I-labled HGF or dHGF were implanted into the back subcutis of mice. At different time intervals, the mouse skin containing the hydrogel implanted was taken out and the corresponding facial site was thoroughly wiped off with the filter paper in the similar way as described above. The radioactivity ratios to the growth factor initially used were expressed as the percentage of remaining activity for *in vivo* growth factor release. On the other hand, for the solution form, after injection of ^{125}I-labeled HGF or dHGF solution into mouse back, the radioactivity around injection site was measured at 2 h, 6 h, 24 h (1 day) and 72 h (3 days).

Complexation of HGF or dHGF with acidic gelatin

Briefly, aqueous solution containing different concentrations of acidic gelatin (100 μl) was mixed at 37°C with 1.25 μg/ml of HGF or dHGF aqueous solution (100 μl). The mixing ratios of growth factor to gelatin were 3 : 10, 3 : 5, 3 : 2, 3 : 1, 3 : 0.5, 3 : 0.2 and 3 : 0.1. The mixed growth factor and gelatin aqueous solutions were left for 24 h to prepare various types of HGF- or dHGF-gelatin complexes.

Proteolytic digestion of HGF and dHGF

The protective effect of the gelatin complexation on the proteolytic digestion of growth factor was evaluated as described previously [18]. Briefly, PBS containing trypsin was added to an aqueous solution of HGF or the HGF-gelatin complex

prepared at 37°C with a HGF/gelatin ratio of 3 : 1. The amounts of trypsin added were 77 or 154 ng/ng protein. Trypsin digestion was allowed to proceed at 37°C for 1 h. At the end of trypsin digestion, trypsin inhibitor of 1.5-times molar quantity was added to the trypsin solution, followed by 1 h incubation at 37°C to block the enzymatic activity of trypsin. As control experiment the similar procedure, except for the trypsin digestion step, was performed for HGF, dHGF and the HGF- or dHGF-gelatin complex. The resulting mixed aqueous solutions were used for electrophoresis assay to evaluate change in molecular mass of HGF or dHGF before and after trypsin digestion.

Electrophoresis assay

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for HGF, dHGF and the HGF- or dHGF-acidic gelatin complex before and after the trypsin digestion. The complex was prepared by mixing for 24 h at 37°C at an HGF or dHGF/gelatin ratio of 3 : 1. The sample solution was added with an equal volume of 0.5 M Tris/HCl-buffered solution (pH 7.0) containing 1 wt% SDS and 50 wt% glycerin and mixed for 5 min. The resulting mixture was subjected to SDS-PAGE with a polyacrylamide gel (10%), and then the resulting gel was fixed with an aqueous solution containing 20 vol% methanol and 7.5 vol% acetic acid and stained with an aqueous solution of Coomassie brilliant blue (CBB) R-250.

Antibody recognition assay

To evaluate the recognition extent of HGF or dHGF by an antibody before and after complexation with the acidic gelatin, an antibody recognition assay was performed based on the ELISA concept. Briefly, 100 μl of aqueous solution containing HGF, dHGF and the HGF- or dHGF-gelatin complexes was added to a well of an ELISA kit of HGF and after that, the conventional assay was performed according to the manual of the ELISA kit. The anti-HGF antibody used in this study detects only 60% of dHGF molecules, unlike HGF. The recognition amount of naked, non-complexed growth factors was expressed as 100% recognition and the amount of HGF or dHGF recognized by the antibody was calculated from the calibration curves which were prepared by different concentrations of each protein solution.

Statistical analysis

All the data were statistically analyzed by Tukey multiple comparison tests and statistical significance was accepted at $P < 0.05$. Experimental results are expressed as the mean $\pm$ the standard deviation of the mean.

RESULTS

Sorption of HGF or dHGF into acidic gelatin

Since the sorption isotherm of HGF or dHGF for gelatin-immobilized agarose beads was of the Langnuir-type, the sorption parameters were calculated based on the Scatchard model. The K_d value of HGF at 37°C was 1.1×10^{-6} M, which is lower than that of dHGF (5.4×10^{-4} M), although the binding ratio was similar (Table 1). This indicates that dHGF has a lower affinity for the gelatin than original HGF.

In vitro *and* in vivo *release of HGF or dHGF from gelatin hydrogel*

Figure 1 shows that the cumulative amount of ^{125}I-labeled HGF or dHGF released from the acidic gelatin hydrogel in PBS. It was observed that dHGF was released faster from the gelatin hydrogel than HGF.

Table 1.
Sorption parameters of HGF or dHGF to acidic gelatin

Sorbate	K_d (M)	HGF/gelatin binding ratio (mol/mol)
HGF	1.1×10^{-6}	2.9
dHGF	5.4×10^{-4}	2.3

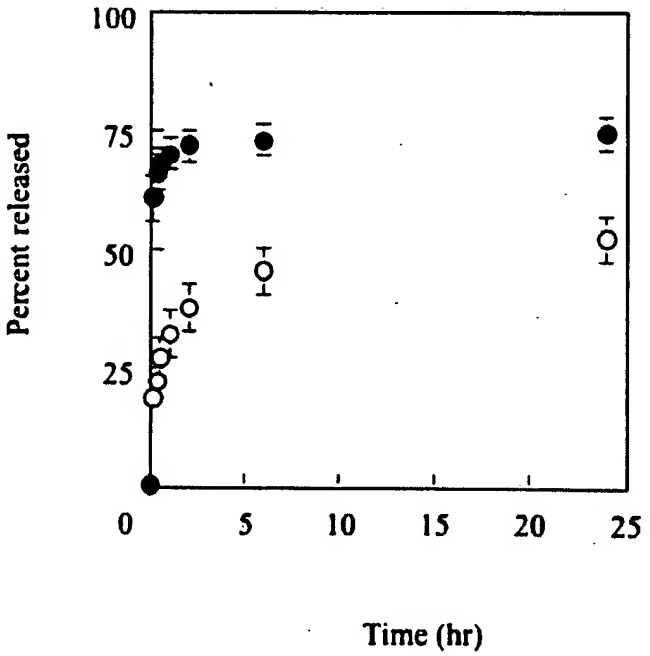

Figure 1. Time-course of the radioactivity release from acidic gelatin hydrogels incorporating ^{125}I-labeled HGF (○) or ^{125}I-labeled dHGF (●) in PBS at 37°C.

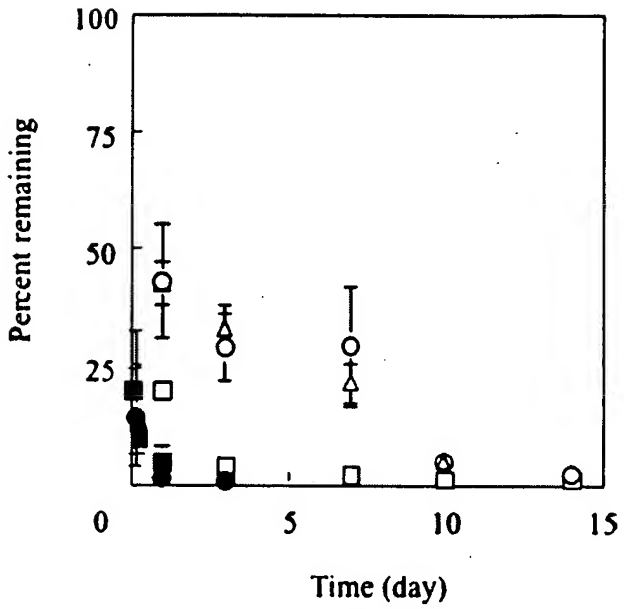

Figure 2. Time-course of the radioactivity remaining after subcutaneous implantation of acidic gelatin hydrogels incorporating ^{125}I-labeled HGF (○) or ^{125}I-labeled dHGF (□), and ^{125}I-labeled acidic gelatin hydrogels (△) and subcutaneous injection of ^{125}I-labeled HGF (●) or ^{125}I-labeled dHGF (■) into the back of mice.

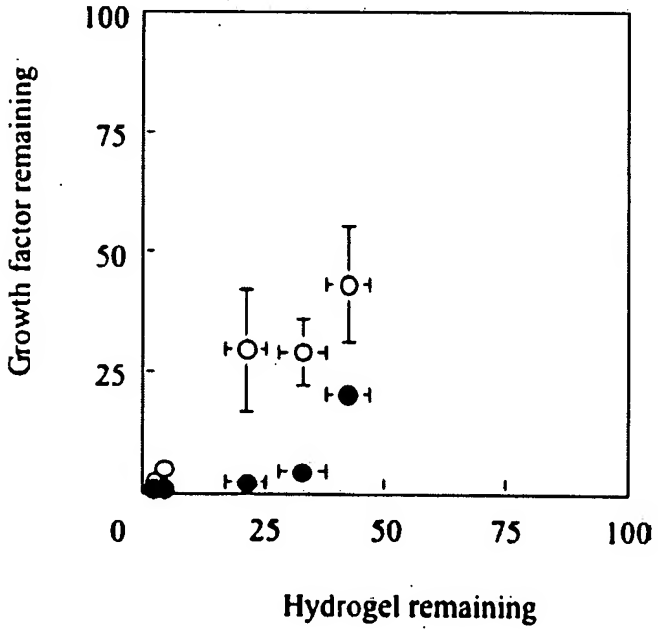

Figure 3. Relationship of the radioactivity remaining between the gelatin hydrogel incorporating ^{125}I-labeled HGF (○) or ^{125}I-labeled dHGF (●) and ^{125}I-labeled hydrogels after subcutaneous implantation into the back of mice.

Figure 2 shows that the decrement patterns of HGF or dHGF radioactivity after subcutaneous implantation of gelatin hydrogels incorporating ^{125}I-labeled HGF or dHGF into the mice show a similar pattern. The residual radioactivity of HGF or dHGF gradually decreased with time. The radioactivity of HGF or dHGF incorporated in the acidic gelatin hydrogel was retained for longer time periods than that of ^{125}I-labeled free HGF and dHGF. However, the radioactivity of dHGF incorporated into the gelatin hydrogel was rapidly decreased and the time profile was different from that of HGF.

Figure 3 shows the radioactivity remaining of HGF or dHGF as a function of that of hydrogel. In the case of HGF, a good correlation in the radioactivity remaining was observed between the protein and the hydrogel of release carrier. The slope of the linear curve was almost 1, which is different from that of dHGF.

Electrophoresis of HGF or dHGF complexed with acidic gelatin before and after trypsin digestion

Figure 4 shows the representative electrophoresis result of HGF, dHGF and the HGF- or dHGF-gelatin complex before and after trypsin treatment. A protein band was detected at the position of an apparent molecular mass of 46 kDa which was attributed to undegraded, original HGF and dHGF. With the increased trypsin

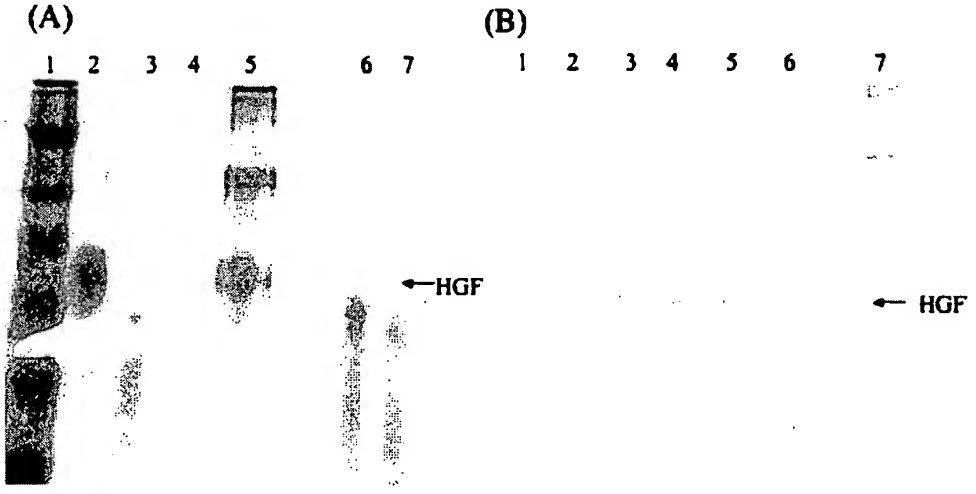

Figure 4. (A) Electrophoretic patterns of free HGF and HGF complexed with the acidic gelatin at 37°C for 24 h after 1 h treatment before and after trypsin at 37°C. Lane 1, marker proteins; lane 2, HGF; lanes 3 and 4, trypsin-treated HGF; lane 5, HGF-acidic gelatin complex; lanes 6 and 7, trypsin-treated HGF-acidic gelatin complexes. The amount of trypsin added to 1 ng of protein was 0.077 (lanes 4 and 7) or 0.154 ng (lanes 3 and 6). (B) Electrophoretic patterns of free dHGF and dHGF complexed with the acidic gelatin at 37°C for 24 h after 1 h treatment before and after trypsin at 37°C. Lanes 1 and 2, trypsin-treated HGF-acidic gelatin complexes; lane 3, dHGF-acidic gelatin complex; lane 4, dHGF; lanes 5 and 6, trypsin-treated dHGF; lane 7, marker proteins. The amount of trypsin added to 1 ng of protein was 0.077 (lanes 2 and 5) or 0.154 ng (lanes 1 and 6).

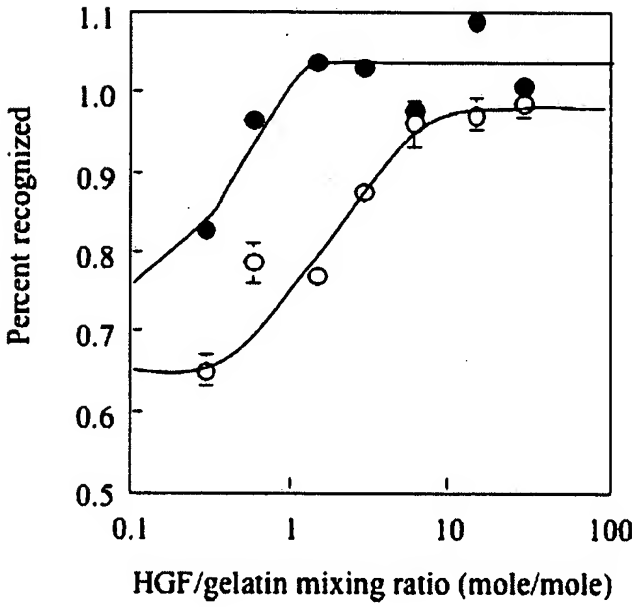

Figure 5. ELISA of HGF (O) or dHGF (●) complexed with the acidic gelatin at 37°C for 24 h at different mixing ratios. The percentage recognition was expressed as the ratio of HGF or dHGF complexed with the gelatin to naked HGF or dHGF, respectively.

concentration, the band of dHGF complexed with the acidic gelatin disappeared. In contrast, for the HGF complexed with the acidic gelatin, the band did not disappear, even at the higher concentration of trypsin. The band of HGF- or dHGF-gelatin complexes before trypsin treatment did not migrate.

Antibody recognition of HGF- or dHGF-gelatin complexes

Figure 5 shows the extent of recognition of HGF- or dHGF-gelatin complexes by the anti-HGF antibody. Irrespective of the type of complex, as the ratio of growth factors increased, the recognition percentage increased up to the same level at which the original growth factor could be recognized. The recognition percentage of HGF complexed with the acidic gelatin gradually decreased for HGF/gelatin ratios of less than 3. However, the recognition percentage of dHGF complexed with the acidic gelatin rapidly decreased only when the dHGF/gelatin ratio was less than 1.

DISCUSSION

This study was designed to evaluate the molecular interaction of HGF or dHGF with acidic gelatin. The Scatchard analysis demonstrates that the interaction of dHGF with acidic gelatin was weaker than that of HGF. Since dHGF is a variant which lacks 5 electronically neutral amino-acid residues (FLPSS), it is considered that there is no difference in the electric nature between the HGF and dHGF

molecules. However, the interaction with the acidic gelatin was different to each other. The interaction difference may be due to other interactions; for example, hydrophobic and hydrogen bonding interactions. The FLPSS sequence contains phenylalanine and leucine residues, of hydrophobic nature, and the serine residue has a hydroxyl group which may contribute to the hydrogen bonding. A recent study has demonstrated that the 5-amino-acid deletion in the first kringle of HGF caused a tertiary structural change [12]. It is conceivable that the different structure causes the extent of gelatin interaction with the HGF and dHGF proteins.

In vitro dHGF was released from the acidic gelatin hydrogel more rapidly than HGF in PBS without any enzyme (Fig. 1). Figure 2 shows that HGF was retained at the implanted site of gelatin hydrogel incorporating HGF for a longer time period than dHGF, while there was a good correlation in the time profile between HGF and gelatin hydrogel retentions. This is because HGF interacts with the acidic gelatin more strongly than dHGF. It is likely that the HGF interacting with the acidic gelatin is released from the gelatin hydrogel only when the hydrogel was degraded to generate water-soluble gelatin fragments. On the other hand, dHGF would be diffused away from the gelatin hydrogel without hydrogel degradation, since the interaction with the acidic gelatin was weaker.

Complexation with acidic gelatin protected HGF from the enzymatic digestion, but not dHGF (Fig. 4). This difference in the protection effect can be explained by the complexation extent. It is possible that firm complexation with the acidic gelatin, due to strong interaction, physically suppresses the direct attack of trypsin to HGF, resulting in reduction of enzymatic digestion of HGF. However, for dHGF, the weaker complexation cannot protect it from the enzyme attack.

A recent study has demonstrated that some antibodies of dHGF recognize the HGF molecule but others do not recognize HGF [12]. The percentage recognition was expressed as the ratio of HGF or dHGF complexed with the gelatin to naked HGF or dHGF, respectively. When the ratio of HGF complexed with the acidic gelatin was less than 3, the amount of HGF recognized by the antibody decreased. On the other hand, at the ratio of dHGF complexed with gelatin more than 1, the dHGF complexed was recognized at the same level as the naked dHGF. This again indicates that the dHGF complexation with the acidic gelatin was weaker than that of HGF. This is due to lower affinity of dHGF for the gelatin.

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EXHIBIT F

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Disassociation of Met-Mediated Biological Responses In Vivo: the Natural Hepatocyte Growth Factor/Scatter Factor Splice Variant NK2 Antagonizes Growth but Facilitates Metastasis

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Hepatocyte growth factor/scatter factor (HGF/SF) stimulates numerous cellular activities capable of contributing to the metastatic phenotype, including growth, motility, invasiveness, and morphogenetic transformation. When inappropriately expressed in vivo, an HGF/SF transgene induces numerous hyperplastic and neoplastic lesions. NK1 and NK2 are natural splice variants of HGF/SF; all interact with a common receptor, Met. Although both agonistic and antagonistic properties have been ascribed to each isoform in vitro, NK1 retains the full spectrum of HGF/SF-like activities when expressed as a transgene in vivo. Here we report that transgenic mice broadly expressing NK2 exhibit none of the phenotypes characteristic of HGF/SF or NK1 transgenic mice. Instead, when coexpressed in NK2-HGF/SF bitransgenic mice, NK2 antagonizes the pathological consequences of HGF/SF and discourages the subcutaneous growth of transplanted Met-containing melanoma cells. Remarkably, the metastatic efficiency of these same melanoma cells is dramatically enhanced in NK2 transgenic host mice relative to wild-type recipients, rivaling levels achieved in HGF/SF and NK1 transgenic hosts. Considered in conjunction with reports that in vitro NK2 induces scatter, but not other activities, these data strongly suggest that cellular motility is a critical determinant of metastasis. Moreover, our results demonstrate how alternatively structured ligands can be exploited in vivo to functionally dissociate Met-mediated activities and their downstream pathways.

Hepatocyte growth factor/scatter factor (HGF/SF) possesses an impressive panoply of biological activities, thereby regulating cellular proliferation and a variety of morphogenetic processes, including cellular migration, extracellular matrix invasion, branching, and tubulogenesis (reviewed in references 15, 21, 29, 51, and 73). Effects of this multifunctional cytokine are all mediated through its cell surface receptor tyrosine kinase (RTK), encoded by the c-*MET* proto-oncogene (4, 12, 32, 37). Upon HGF/SF binding, MET engages a number of SH2-containing signal transducers, including phosphotidylinositol 3-kinase, phospholipase C-γ, Stat3, Grb2, and the Grb2-associated docking protein Gab1, and indirectly activates the Ras–mitogen-activated protein kinase (MAPK) pathway (39, 40, 69, 70). Typically, HGF/SF is produced in cells of mesenchymal origin, influencing Met-expressing embryonic and adult epithelium through a paracrine mechanism (19, 59, 64). Gene targeting studies have demonstrated that activation of signaling pathways downstream of Met is essential for development of murine skeletal muscle, liver, and placenta (3, 53, 67). In accordance with its various effects on cultured cells, HGF/SF is thought to regulate epithelial-mesenchymal conversion and migration of myogenic precursor cells in vivo.

Chronic MET activation induces the genesis and, more significantly, progression of a multitude of human and murine tumors, including melanomas (for example, see references 2, 13, 14, 22, 33, 41, 43, 44, 46, 47, and 65). MET activation can be achieved through coexpression of HGF/SF, resulting in the creation of an autocrine signaling loop (2, 13, 43, 45, 65). In addition, critical genetic evidence for a role for c-*MET* in human cancer has come from the discovery that activating c-*MET* mutations are associated with hereditary papillary renal carcinoma (24, 54, 74). As during embryogenesis, a number of activities ascribed to HGF/SF and Met activation undoubtably contribute to the manifestation of the full metastatic phenotype. These include stimulation of angiogenesis, degradation of local extracellular matrix, production of cell adhesion molecules, migration into vessels and tissues, and colonization at a distant site (reviewed in references 29 and 48).

HGF/SF shows a 38% overall sequence similarity with plasminogen (15) and a 45% identity to HGF-like/macrophage-stimulating protein at the amino acid level (17, 72). The 92-kDa HGF/SF possesses several recognizable structures, which are shared by all family members, including the presence of an enzymatically inactive serine protease domain in the β chain, and an N domain and four kringle domains in the α chain (Fig. 1A). Kringles are highly conserved, three-disulfide, triple-loop polypeptides thought to participate in protein-protein interactions (reviewed in reference 66). HGF/SF mRNA can undergo alternative splicing to create truncated isoforms (Fig. 1A), capable of binding to the HGF/SF receptor with relatively high affinity. Historically, defining the biological activities associated with these variants has been somewhat elusive and a point of contention in the field. One natural variant consisting of the N domain and the first two kringle domains, designated NK2, was originally found to be incapable of stimulating the growth of cultured human mammary epithelial cells but instead antagonized HGF/SF-induced mitogenesis (6, 30). However, NK2 was later reported to act as a partial agonist, able to scatter certain cultured epithelial cells (18, 60). More recently, NK2 was shown to be incapable of triggering induction of tissue inhibitor of metalloproteases 3, urokinase-type plasmin-

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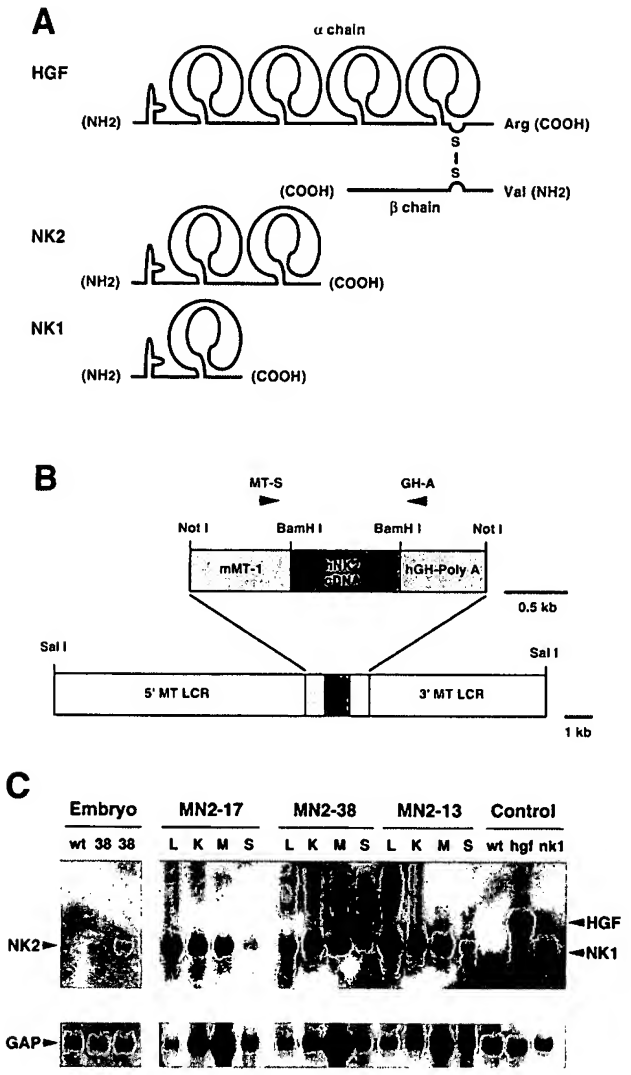

FIG. 1. Structure and expression of NK2. (A) Schematic comparison of HGF/SF (designated as HGF in this and all other figures) and its natural splice variants NK2 and NK1. Each isoform contains a single so-called N domain at the amino terminus, and either four, two, or one kringle domain, as shown. However, only HGF/SF is processed into two chains, the β chain containing an enzymatically inactive serine protease domain. (B) The *SalI-SalI* NK2 transgene construct contained the human NK2 cDNA, the mouse MT gene promoter (mMT-1) and 5′ and 3′ flanking sequences (MT LCR), and the hGH poly(A) signal. Mice harboring the NK2 transgene were identified by PCR using primers MT-S and GH-A, as indicated. (C) Analysis of NK2 transgene expression in mouse tissues by Northern blot hybridization. For embryonic expression (three-lane panel at left), wild-type (wt) embryos were harvested at E16.5, and transgenic embryos from line MN2-38 (38) were harvested at E14.5 (middle lane) and E16.5 (right lane). Adult (2-month-old) tissues from three independently generated lines, MN2-17, MN2-38, and MN2-13, were studied. Tissues analyzed included liver (L), kidney (K), skeletal muscle (M), and skin (S). The control lanes at far right show expression of HGF/SF sequences in livers of wild-type and HGF/SF and NK1 transgenic mice. Following hybridization with a human NK2 cDNA probe (top panels), the filter was stripped and rehybridized with a control GAP cDNA probe (bottom panels).

ogen activator proteolysis, invasion, or tubulogenesis in some cells (5, 23, 31). Interestingly, a unique bivalent monoclonal antibody against a non-binding-site epitope of the extracellular domain of human HGF/SF was, like NK2, found to stimulate cell motility but no other Met-associated activity (42). A second truncated HGF/SF, NK1, was first artificially engineered to consist of the N domain and a single kringle domain but was later found to occur naturally in mouse cells as well (9, 28, 60). NK1 was also originally reported to possess activities antagonistic to HGF/SF in terms of mitogenesis (28) but later found to stimulate mitogenic and motogenic activities (9). Schwall et al. (55) have provided evidence suggesting that the presence of cell surface heparan sulfate proteoglycans can facilitate NK1 mitogenic activity by inducing ligand dimerization. An artificial four-kringle mutant, NK4, was reported to inhibit the mitogenic, motogenic, and morphogenic activities of HGF/SF in vitro (10). Taken together, these data indicate that the in vitro biological activities of these HGF/SF variants are context dependent and greatly influenced by the target cell and the culture conditions in which those cells are grown; moreover, they stand as a testament to the requirement for in vivo models to assess their bona fide activities.

To this end, we have generated a series of transgenic mice in which either HGF/SF, NK1, or NK2 was broadly expressed using a mouse metallothionein (MT) promoter and associated locus control regions (LCRs) to regulate transcription. Previously, we demonstrated that ectopic expression of HGF/SF had pleiotropic phenotypic consequences, including enhanced liver growth and regeneration, progressive renal disease characterized by glomerulosclerosis, disruption of the olfactory mucosa, aberrant appearance of skeletal muscle in the central nervous system, patterned hyperpigmentation and aberrant localization of melanocytes in the dermis and epidermis, precocious mammary lobuloalveolar development, and susceptibility to diverse tumorigenesis (52, 61–63) (Fig. 2). More recently, we reported that mice expressing an NK1 transgene exhibited a remarkably similar array of phenotypes, albeit with reduced severity, indicating that NK1 is a partial agonist of HGF/SF in vivo (20). In striking contrast, here we demonstrate that NK2 can antagonize most of the phenotypic consequences of HGF/SF expression in mice harboring both transgenes. However, by employing various genetically modified host mice as tumor transplant recipients, we show that NK2 alone retains an impressive ability to facilitate the metastasis of melanoma cells expressing high levels of Met.

MATERIALS AND METHODS

Generation and identification of transgenic mice. NK2 transgenic mice were generated on an albino FVB/N genetic background employing the expression construct used previously for the HGF/SF and NK1 transgenic mice. Expression of the human NK2 cDNA was placed under the control of the mouse MT-1 promoter. The construct included the human growth hormone (hGH) polyadenylation site [poly(A)] and the 5′ and 3′ flanking regions of mouse MT genes (Fig. 1B). These contain LCRs conferring copy-number-dependent and integration-site-independent transgene expression (36). NK2 transgenic mice were identified by PCR using as template tail genomic DNA and the following primer set: MT-S (5′-ACTCGTCCAACGACTATA-3′), specific to the MT promoter region, and GH-A (5′-AACTTCCAGGGCCAGGAGA-3′), specific to the hGH-poly(A) sequence. HGF/SF transgenic mice were identified by PCR using the following primer set: HGF315 (5′-AGTTATGGTTGTACAATCCCTGAAAA GA-3′), specific to the β chain of mouse HGF/SF sequence, and GH-A. NK1 transgenic mice were identified by PCR using the following primer set: MT-S and HGF292 (5′-CTGAGGAATGTCACAGACTTCGTA-3′), specific to the first kringle domain sequence of mouse HGF/SF cDNA. Diagnostic PCR products for the presence of the NK2, HGF/SF, and NK1 transgenes were 1,019, 451, and 709 bp, respectively. Where noted, mice were maintained on 25 mM $ZnSO_4$ in their drinking water. All mouse work was performed in accordance with the Guide for the Care and Use of Laboratory Animals (33a).

Histopathological assessment and liver growth analysis. For routine histopathological analysis, mouse tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Metastatic melanomas were visualized using an anti-mouse tyrosinase-related protein 1 (TRP1) antibody, αPEP1 (35), a gift from Vincent Hearing, National Cancer Institute, Bethesda, Md. For comparative analysis of hepatocyte proliferation in vivo, five to seven 1.5-month-old mice of each genotype that had been maintained on $ZnSO_4$ water were given intraperitoneal injections of bromodeoxyuridine (BrdU), according to the manufacturer's instructions (Amersham Life Science; RPN201). After 2 h, all mice were euthanatized and their liver tissues were fixed in 70% ethanol. BrdU incorporation was then detected

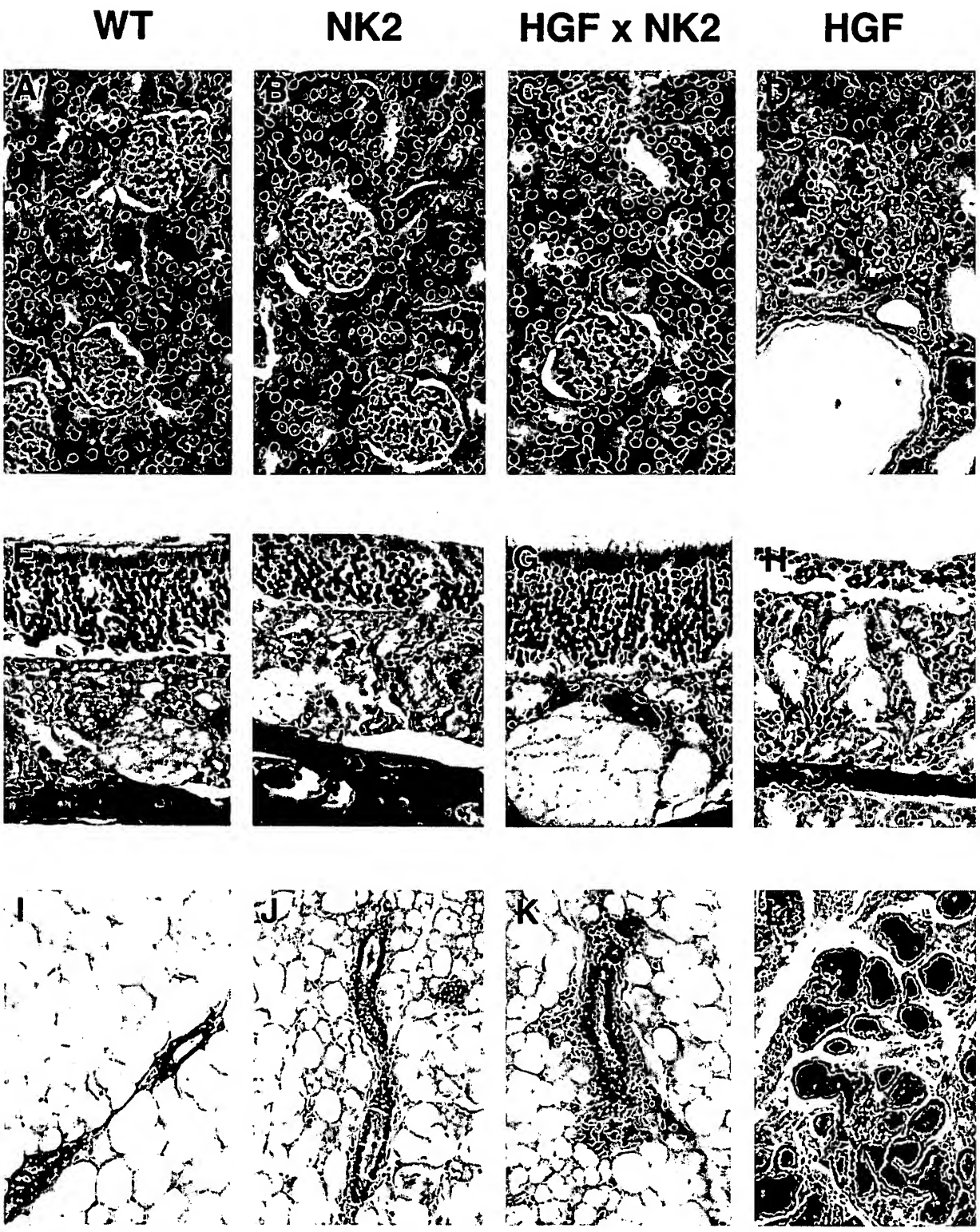

FIG. 2. NK2 extinguishes the phenotypic consequences of ectopic HGF/SF expression in bitransgenic mice. Shown are panels of tissues, including kidney (A to D), olfactory mucosa (E to H), and virgin mammary gland (I to L), from wild-type (A, E, and I), NK2 transgenic (B, F, and J), HGF/SF-NK2 bitransgenic (C, G, and K), and HGF/SF transgenic (D, H, and L) mice. Tissues shown are from mice 2.5 months of age. Note that bitransgenic tissues resemble wild-type tissues and do not contain the pathological features characteristically evident in HGF/SF transgenic animals.

immunohistochemically (56), and labeled hepatocyte nuclei from between 422 and 589 high-power light microscope fields (400×) for each genotype were scored. For determination of liver growth, between 18 and 27 female mice of each genotype between 1.5 and 3.0 months of age were used. Exposure to $ZnSO_4$ water had no overt effect on liver mass, so data from zinc-treated and non-zinc-treated animals were combined.

Analysis of RNA. NK2 transgene expression in selected adult (2-month-old) tissues was assessed 6 h after intraperitoneal injection of 5 mg of $ZnCl_2$ per kg

of body weight. To compare transgene expression with liver weight/body weight ratios, total RNA was isolated from 1.5-month-old transgenic and bitransgenic female mice maintained on 25 mM $ZnSO_4$ water. For fetal expression, E14.5 and E16.5 mouse embryos were used for RNA isolation. Total RNA was prepared using guanidine thiocyanate, as described previously (25). For Northern blot analysis, 15 μg of total RNA was resolved on a denaturing 1% agarose-formaldehyde gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was prehybridized and hybridized at 42°C in a solution whose contents included 50% formamide and 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), washed, and subjected to autoradiography (25). The 2.2-kbp mouse HGF/SF cDNA probe was synthesized by PCR, as described previously (62). The 636-bp mouse NK2 probe, covering only the first and second kringle domain of the HGF/SF cDNA, was synthesized by PCR using the following primer set: HGF313S (5'-GAGTGTGCCAACAGGTGTATCAGG-3') and HGF291 (5'-AATTGCACAATACTCCCAAGGGGT-3'). For analysis of MT expression, a 355-bp *Bam*HI mouse MT-1 cDNA fragment was used as hybridization probe (generously provided by Richard Palmiter, University of Washington, Seattle). To control for RNA loading and transfer variation, filters were routinely rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAP) cDNA probe (35).

Cell culture and transplantation. Both the mouse cell lines, 37-32 and 37-7, were derived from neoplasms arising in HGF/SF transgenic mouse line MH-37 (35, 62). Both lines were maintained in Dulbecco modified Eagle medium (DMEM) (Gibco) supplemented with 15% fetal bovine serum (Gibco), 100 IU of penicillin (Gibco) per ml, 100 μg of streptomycin (Gibco) per ml, 2 mM L-glutamine (Gibco), 5 μg of insulin (Upstate Biotechnology Inc.) per ml, and 5 ng of epidermal growth factor (Upstate Biotechnology Inc.) per ml, and incubated in 5% CO_2 at 37°C. Subcutaneous tumors were produced by injection of 10^6 cells in 0.3 ml of DMEM under the back skin of 2- to 3-month-old wild-type, HGF/SF transgenic, NK2 transgenic, or HGF/SF-NK2 bitransgenic male and female mice. Tumor diameters were measured every 3 days using a caliper, and tumor volumes were calculated according to the formula $V = a \times b^2/2$. Rates of tumor growth were determined based on 3-day intervals. For the experimental metastasis assay, 10^5 or 10^6 cells in 0.3 ml of DMEM, as indicated, were intravenously injected via the tail vein into 2- to 5.5-month-old male and female HGF/SF transgenic, NK1 transgenic, NK2 transgenic, and wild-type mice. The conclusions from the metastasis assay were essentially the same whether 10^5 or 10^6 melanoma cells were injected. Gross tumor numbers were obtained by visual inspection of liver, spleen, kidneys, lungs, diaphragm, and pleural cavity in mice euthanatized 18 to 25 days posttransplantation. Microscopic quantification of metastasis was performed on representative formalin-fixed, H&E-stained sections of all liver lobes from two to eight representative animals between 1.5 and 2.5 months of age; all mice in this study were euthanatized and analyzed 21 days posttransplantation. For tumor size determination, 725, 98, and 65 tumors were measured from representative sections of each liver lobe from NK2, NK1, and HGF/SF mice, respectively. Statistical analysis was performed using the Student *t* test.

Analysis of Met and Met activity. Quantification of Met and Met tyrosine phosphorylation was performed as described previously (35). Lysates were prepared from 37-32 cells treated for 10 min at 37°C with the factors indicated (HGF/SF, 100 ng/ml; NK2, 300 ng/ml). Cultured cells were solubilized in RIPA buffer (50 mM Tris [pH 7.4], 50 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM sodium pyrophosphate [Sigma], 50 mM sodium fluoride [Sigma], 1 mM sodium orthovanadate [Sigma], 1 mM phenylmethylsulfonyl fluoride [Boehringer Mannheim], 10 μg of leupeptin [Boehringer Mannheim] per ml, 10 μg of pepstatin [Boehringer Mannheim] per ml, and 10 μg of aprotinin [Boehringer Mannheim] per ml). Equivalent amounts of the resulting lysates were incubated with anti-Met antibody (Santa Cruz Biotechnology) for 2 h. Following addition of GammaBind G Sepharose (Pharmacia Biotech) and washing in RIPA buffer, samples were fractionated by reducing sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel electrophoresis (PAGE). After electrophoretic transfer to Immobilon-P membranes (Millipore), filters were blocked and incubated with anti-Met antibody (Santa Cruz Biotechnology) overnight. Met was visualized by incubation with anti-rabbit antibody conjugated to horseradish peroxidase, followed by enhanced chemiluminescence (ECL; Amersham). After stripping, filters were reblocked and incubated overnight with a phosphotyrosine monoclonal antibody (Upstate Biotechnology).

To determine the comparative effects of HGF/SF and NK2 on Met-induced MAPK activity, Western blotting for anti-active MAPK was performed as described previously (11). Lysates, prepared after exposure of 37-32 cells to either HGF/SF or NK2 (as above), were fractionated by SDS–12% PAGE, transferred to Immobilon-P membrane, and probed with anti-phospho-MAPK antibody (New England Biolabs) as per the manufacturer's instructions. Positive staining was detected by ECL (Amersham).

Analysis of HGF/SF and NK2. Mouse liver lysates (25 μg of total protein per sample per lane) were fractionated by SDS–10% PAGE and electrophoretically transferred to Immobilon-P membrane. Membranes were blocked with bovine serum albumin, probed with anti-human HGF/SF (N-17; Santa Cruz Biotech) in 0.1% bovine serum albumin–0.05% NP-40–phosphate-buffered saline, and detected by ECL (Amersham). A series of standards of purified mouse HGF/SF and human NK2 run in the same gel as the liver samples allowed a quantitative assessment of HGF/SF and NK2 levels. Purified mouse HGF/SF was a generous gift from Ermanno Gherardi, MRC Center, Cambridge, United Kingdom; human NK2 was prepared as described previously (60). These standards revealed that the anti-human HGF/SF antiserum used for immunoblot analysis recognized human HGF/SF with greater overall sensitivity than it recognized mouse HGF/SF, although both were readily detectable. NK2 levels in sera were quantified using an enzyme-linked immunosorbent assay, as described previously (55).

RESULTS

Mice expressing NK2 are healthy and exhibit no overt hyperproliferative lesions. To determine the in vivo biological activities of the natural HGF/SF splice variant, NK2, the human cDNA was placed under the transcriptional control of the mouse MT gene promoter and LCRs (Fig. 1B), and the resulting expression vector was used to make four lines of NK2 transgenic mice. An identical MT expression construct was used in our previously described HGF/SF and NK1 transgenic mice (20, 61), so as to allow a direct comparison of phenotypic consequences of expression of the various HGF/SF isoforms. Northern blot hybridization (Fig. 1C) was used to demonstrate that the MT-NK2 transgene, like the MT-HGF/SF and MT-NK1 transgenes, was highly and broadly expressed in three lines, two of which (MN2-17 and MN2-38) were chosen for further analysis. In all experiments presented here, results obtained using these two lines were indistinguishable. Transgene expression was also assessed during development in line MN2-38. As with the MT-HGF/SF transgene (61), the MT-NK2 transgene was clearly active in E14.5 and E16.5 embryos (Fig. 1C). An enzyme-linked immunosorbent assay showed that NK2 levels in the serum of MN2-38 mice averaged 27 ng/ml; wild-type control serum had an NK2 level less than the 7.8-ng/ml limit of detection. Previously, HGF/SF transgenic mouse serum was found to contain an average of 16.4 ng of HGF/SF per ml compared to wild-type levels of 3.9 ng/ml (61).

In contrast to mice bearing either the MT-HGF/SF or MT-NK1 transgene, NK2 transgenic mice failed to exhibit overt abnormal phenotypes. No hyperplastic lesions were observed in the kidney or olfactory mucosa (Fig. 2B and F), and no anomalies were associated with the mammary gland (Fig. 2J) or skeletal muscle (data not shown). NK2 mice did not experience gastrointestinal obstruction or progressive renal disease, which is highly characteristic of HGF/SF mice (Fig. 2D). The liver was not enlarged; in fact, when expression of the MT-NK2 transgene was stimulated by exposing juvenile mice to zinc-containing water, the weight of the liver relative to the body appeared to be slightly reduced. When crossed with the pigmented strain C57BL/6, first-generation MN2-38 and MN2-17 transgenic mice were found to exhibit no overt hyperpigmentation. However, histopathological analysis of their skin revealed the occasional presence of pigment cells outside the normal confines of the hair shaft, in the dermis and epidermis (data not shown). This ectopic pigment cell localization was more obvious in line MN2-38, which was characterized by higher transgene expression in the skin (Fig. 1C). Such aberrant pigment cell localization was not observed in wild-type animals.

NK2 antagonizes HGF/SF-induced pathology in bitransgenic mice. The small reduction in liver size observed in zinc-treated NK2 transgenic mice raised the possibility that NK2 was capable of inhibiting HGF/SF-mediated hepatocyte proliferation in vivo. To further test this hypothesis, bitransgenic mice harboring both the MT-HGF/SF and MT-NK2 transgenes were generated. Figure 3 shows that NK2 expression in all bitransgenic mice reduced to nearly normal levels the anomalous liver growth associated with the constitutive activation of Met in HGF/SF transgenic hepatocytes (52). Moreover, analysis of BrdU incorporation revealed that the labeling index

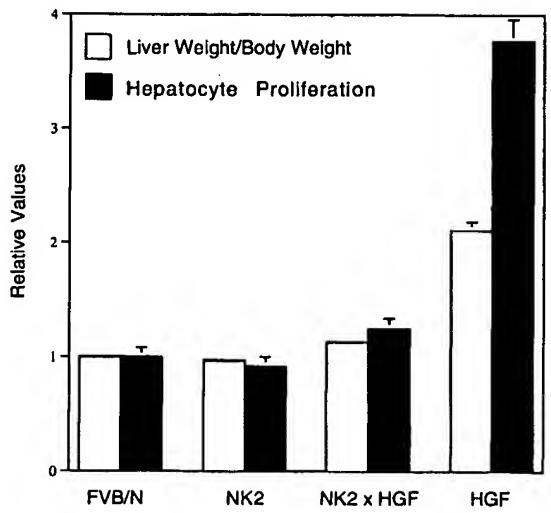

FIG. 3. NK2 inhibits the proliferative effects of HGF/SF on hepatocytes. Shown are mean liver weight/body weight ratios (white bars) and hepatocyte labeling indices (black bars) from wild-type (FVB/N), NK2 transgenic, NK2-HGF/SF bitransgenic, and HGF/SF transgenic livers. All values were normalized to wild type (set at 1.0), and error bars represent standard errors of the means. For both liver size and hepatocyte proliferation, the P value is <0.0001 in NK2-HGF/SF bitransgenic versus HGF/SF transgenic mice.

of bitransgenic hepatocytes was significantly decreased relative to HGF/SF transgenic hepatocytes (Fig. 3). The presence of the MT-NK2 transgene could be inhibiting hepatocyte proliferation in bitransgenic mice by specifically antagonizing HGF/SF activity or by squelching expression of the MT-HGF/SF transgene through competition for MT-specific transcription factors. To distinguish between these two possibilities, the structure and activity of these two transgenes were compared. Southern blot analysis revealed that the two MT-driven transgenes were equivalently represented in terms of copy number (data not shown). Northern blot analysis demonstrated that at the level of RNA, expression of the two transgenes and the endogenous MT gene was coordinately regulated in bitransgenic hepatocytes (Fig. 4A), indicating that there was no shortage of MT-specific transcription factors for which the two transgenes would have to compete. The same result was also seen in livers from bitransgenic juvenile mice that were exposed to water containing zinc (data not shown). Western blotting was then utilized to quantify the relative levels of HGF/SF and NK2 protein in transgenic and bitransgenic liver extracts. Based on comparisons with recombinant standards, it can be estimated that, in 25 μg of transgenic liver extract, HGF/SF and NK2 are represented at levels between 2 and 4 ng and 5 and 10 ng, respectively (Fig. 4B). Thus, the 33-kDa NK2 appears to be present in the bitransgenic livers in about a sevenfold molar excess relative to the 92-kDa HGF/SF. Figure 4B also demonstrates that NK2 is not expressed at the expense of HGF/SF and confirms that the normal liver weight/body weight ratios characteristic of bitransgenic mice are not caused by diminution of HGF/SF levels. These data support the contention that NK2 can antagonize certain HGF/SF-mediated activities at the level of ligand-receptor interaction in vivo, as has been shown in vitro (6, 11, 31).

Prompted by these liver results, we analyzed other tissues from HGF/SF-NK2 bitransgenic mice up to 6 months of age. In contrast to HGF/SF transgenic mice (Fig. 2D, H, and L), in HGF/SF-NK2 bitransgenic mice the kidney exhibited little or no glomerulosclerosis or tubular hyperplasia; the olfactory mucosa was overtly normal, with no sign of olfactory gland hyperplasia or nervous depletion; and virgin mammary epithelium demonstrated no obvious precocious alveolar development (Fig. 2C, G, and K). These results indicate that the pathological consequences of chronic, HGF/SF-mediated Met activation associated with anomalous cellular proliferation can be effectively antagonized in vivo by the splice variant NK2.

NK2 facilitates metastasis but not growth of Met-overexpressing malignant melanoma cells in vivo. Previously, we determined that cultured 37-32 cells, a melanoma line established from HGF/SF transgenic mice and overexpressing both the transgene and endogenous c-*met*, could be growth inhibited up to, but not more than, 60% in minimal medium supplemented with recombinant NK2 to levels in 100-fold excess (0.3 to 1.0 μg/ml) of those that can be achieved in serum, in vivo (35). Unlike HGF/SF, NK2 is incapable of inducing robust Met autophosphorylation or MAPK in these melanoma cells (Fig. 5). To ascertain the relative effect of NK2 on growth and metastasis of 37-32 cells in vivo, transgenic lines of mice overexpressing various HGF/SF isoforms were exploited as genetically modified hosts for transplantation challenge. Initially, 10^6 melanoma cells were injected subcutaneously into syngeneic wild-type FVB/N, transgenic HGF/SF, transgenic NK2, or HGF/SF-NK2 bitransgenic host mice. Figure 6 shows that melanomas grew in the NK2 transgenic hosts nearly as well as in the wild-type mice. However, when transplanted subcutaneously into HGF/SF mice, melanomas became palpable much earlier and grew at an accelerated rate relative to those in either wild-type or NK2 mice. Significantly, the time of appearance and rate of growth of tumors transplanted into bitransgenic host animals expressing both NK2 and HGF/SF were no different than those for either the wild-type or NK2 single transgenic hosts. Together, these data indicate that, while NK2 could not effectively disrupt the HGF/SF-Met autocrine signaling loop driving baseline 37-32 melanoma growth, this variant was able to completely antagonize the paracrine-enhanced in vivo growth associated with overexpression of the HGF/SF transgene originating from genetically modified host mouse tissues.

The 37-32 melanoma cells, derived on an FVB/N inbred genetic background, were shown to be highly metastatic to a number of nude mouse tissues but with a clear partiality for liver (35). To determine the specific effect of the host-generated HGF/SF isoforms on metastasis, 37-32 melanoma cells were introduced intravenously into syngeneic wild-type and HGF/SF, NK1, and NK2 transgenic mice. Gross metastatic colonization by 37-32 cells of the liver and other organs was elevated in HGF/SF transgenic mice relative to that in wild-type controls (Table 1; Fig. 7A), reminiscent of the heightened growth response of these same melanoma cells when transplanted subcutaneously into HGF/SF transgenic mice (Fig. 6). Gross metastatic efficiency of 37-32 cells was enhanced in NK1 transgenic hosts as well (data not shown). When this experimental metastasis assay was repeated with another cell line containing barely detectable levels of HGF/SF and Met (HGF/SF transgenic mouse-derived line 37-7 [35]), the incidence of gross metastasis to a variety of target organs in both HGF/SF and NK2 transgenic host mice was not significantly different from that for wild-type mice (Table 1). This result suggests that host-generated HGF/SF isoforms were acting directly through responsive melanoma cells and not by creating a more permissive host.

Having established the positive impact of host-generated HGF/SF on metastasis of high-Met melanoma cells, the effects of host-generated NK2 were next considered. Remarkably, rather than antagonizing metastasis, as it did growth, host-generated NK2 dramatically stimulated the incidence of gross

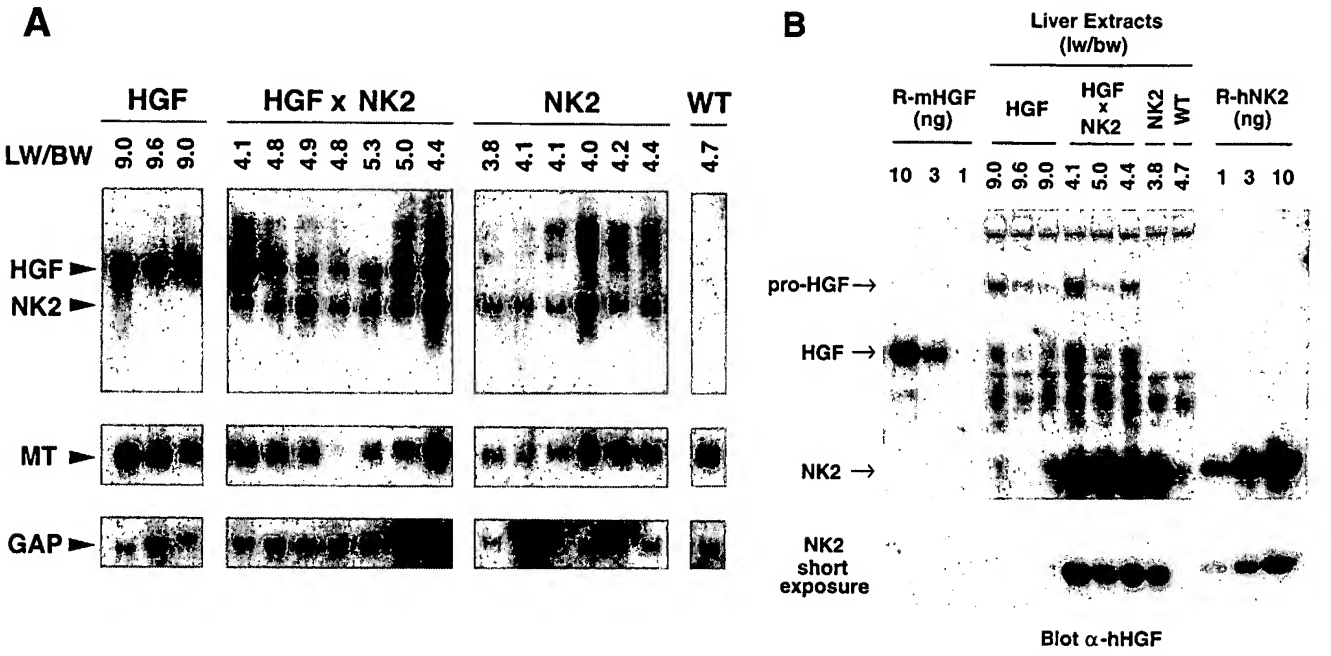

FIG. 4. Comparative transgene expression in livers of HGF/SF and NK2 transgenic and HGF/SF-NK2 bitransgenic mice. (A) Northern blot analysis of total liver RNA (15 μg/sample) using as probe mouse sequences equivalent to NK2 (top panels), mouse MT cDNA (middle panels), or mouse GAP cDNA (bottom panels). Numbers at the top indicate liver weight/body weight ratios (LW/BW). All mice were 1.5 months of age. (B) Quantitative Western blot analysis of mouse HGF/SF and human NK2 transgenic mouse liver extracts (25 μg/sample) using an anti-human HGF/SF antibody. Arrows mark positions of unprocessed pro-HGF/SF, processed HGF/SF, and NK2. Liver weight/body weight ratios (lw/bw) are shown at top. Recombinant mouse HGF/SF (R-mHGF) and human NK2 (R-hNK2) standards of known amounts (in nanograms) are displayed on the extreme left and right, respectively. A short exposure shown at bottom permits quantification of NK2 levels in liver extracts. In general, RNA and protein results in panels A and B, respectively, are in accord.

hepatic metastasis of 37-32 cells, achieving levels that were at least as high as those found in HGF/SF transgenic hosts (Table 1; Fig. 7A). This gross observation was confirmed microscopically and quantified by subjecting several experimental livers to histopathological analysis (Fig. 7B). The efficiency of microscopic metastasis to liver was approximately ninefold greater in NK2 transgenic hosts than in their wild-type counterparts ($P < 0.0005$) and equal to or greater than that in either HGF/SF or NK1 mice (Table 1 and data not shown). In contrast, the average size of the metastatic tumors in the NK2 mouse livers appeared to be equivalent to that in wild type and reduced compared to either NK1 or HGF/SF mice (Fig. 7A and B). Morphometric quantification confirmed that, although the number of liver metastases was enhanced by NK2 as for the other isoforms, growth at the colonization site relative to that for HGF/SF and NK1 was significantly reduced (Fig. 7C). Kidney metastases were also smaller and therefore less conspicuous at both the gross and the microscopic level in NK2 transgenic hosts compared to those in HGF/SF mice (data not shown); however, kidney metastases were present in significantly greater numbers relative to wild-type hosts as well (Table 1).

DISCUSSION

The ability of the HGF/SF alternative splice variants, NK1 and NK2, to function in vitro as HGF/SF agonists or antagonists appears to be contextual, depending on the cell type and the conditions under which it is cultured (6, 9, 18, 23, 28, 30, 31, 34, 55, 58, 60). These isoforms have been detected in mice and humans, and yet their in vivo function is unknown. Recently, we demonstrated that NK1, when broadly expressed in mice, induces all phenotypes observed in HGF/SF transgenic mice, although with reduced severity (20). From this result, we concluded that NK1 acts in vivo as a partial agonist of HGF/SF. In the present study, we show that the in vivo behavior of NK2 is distinct from that of NK1, and more complex. With the exception of relatively mild ectopic localization of melanocytes outside the hair follicles, NK2 transgenic mice exhibited no overt phenotypic abnormalities. However, NK2 effectively mitigated the constellation of HGF/SF-mediated lesions in HGF/SF-NK2 bitransgenic mice, including liver enlargement and elevated hepatocyte proliferation, olfactory gland hyperplasia and mucosal disorganization, renal tubular hyperplasia and subsequent glomerulosclerosis, alveolar mammary hyperplasia, and hyperpigmentation. These lesions are generally attributable to dysregulated cellular growth. The observed coordinate regulation of expression of the two MT-driven transgenes, at the level of both RNA and protein, in bitransgenic livers indicated that amelioration of the phenotypes associated with HGF/SF overexpression in these animals was not the simple consequence of transcription factor competition, or squelching. Instead, NK2 protein itself appeared to effectively antagonize HGF/SF-induced, Met-mediated mitogenic signaling in vivo.

Remarkably, however, when introduced intravenously into the tail vein of NK2 transgenic mice, 37-32 melanoma cells exhibited a ninefold enhancement in efficiency of metastasis to the liver, their preferred site, relative to wild-type host mice of the same age, sex, and genetic background. Moreover, the incidence of metastasis in NK2 animals was at least as high as that observed in either HGF/SF or NK1 transgenic hosts, indicating that NK2 functions in vivo as a potent agonist of Met-driven metastatic dissemination. Notably, however, the average mass of liver metastases from these NK2 transgenic hosts was reduced approximately five- or threefold relative to those arising over the same time in HGF/SF or NK1 animals,

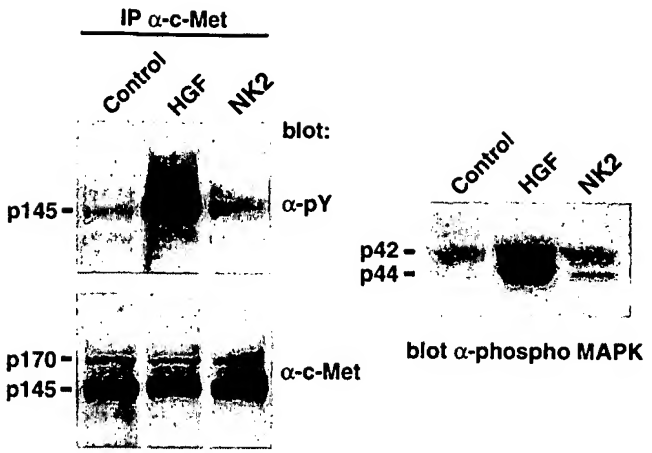

FIG. 5. Quantification of Met and Met activity in melanoma cells. Extracts prepared from 37-32 melanoma cells treated with either nothing (control), HGF/SF, or NK2 were immunoprecipitated (IP) with anti-Met antibody and subsequently probed with either anti-phosphotyrosine (α-pY, top left) or anti-Met (α-c-Met, bottom left) antibodies. Extracts were also directly probed with anti-phospho-MAPK antibody (right). Molecular masses in kilodaltons are shown. Note that, relative to NK2, HGF/SF induces phosphorylation of Met and MAPK without altering the levels of Met.

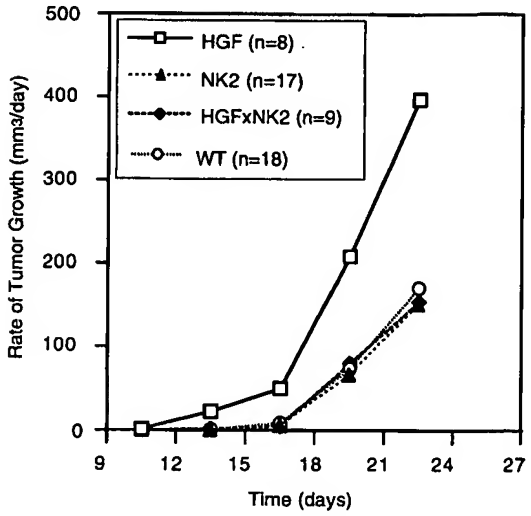

FIG. 6. NK2 antagonizes paracrine, but not autocrine, HGF/SF-induced subcutaneous melanoma growth. One million 37-32 melanoma cells were injected under the back skin of 2- to 3-month-old wild-type (WT), HGF/SF transgenic, NK2 transgenic, and HGF/SF-NK2 bitransgenic mice; tumor sizes were measured; and growth rates were calculated.

respectively, indicating that, in this experimental model of metastasis, NK2 was incapable of stimulating melanoma growth in the manner demonstrated by host-generated HGF/SF. This metastatic behavior was mirrored in other organs as well. Together, these findings clearly show that the activities associated with HGF/SF, and mediated by a single RTK, Met, can be functionally dissociated in vivo. Furthermore, the same isoform can apparently serve in vivo as both HGF/SF agonist and antagonist.

How can NK2 simultaneously induce agonistic and antagonistic activities, and why do NK1 and NK2 evoke such different in vivo responses? Despite the availability of a relatively detailed blueprint of Met signaling pathways and their integral components, the mechanistic basis by which various HGF/SF isoforms differentially elicit Met-mediated cellular responses is not yet understood. It is assumed that ligand-induced dimerization triggers the activation-autophosphorylation of generic RTKs, including Met (reviewed in reference 71), and that processed, two-chain HGF/SF binds Met, either as a monomer or as a dimer, inducing a conformational shift toward a stabilized active receptor configuration (reviewed in reference 7). The recently resolved crystal structure of NK1 suggests assemblage as a homodimer capable of simultaneously engaging two Met receptors and provides a rationale for the agonism demonstrated by NK1 (8, 68). Interaction with endogenous glycosaminoglycans may be exceedingly important in realizing the agonistic behavior of this variant in vivo (8, 9, 52, 55, 68). If it is a pure antagonist, the behavior of NK2 could be explained through its ability to compete with HGF/SF for receptor binding, without inducing the appropriate activating conformational change in Met. However, we show in this report that NK2 does not behave as a pure antagonist in vivo. Although all

TABLE 1. Incidence of metastasis and organ site selection of malignant cells in host transgenic mice expressing HGF/SF or its splice variants[a]

Host genotype	Analysis	High-Met 37-32 cells			Low-Met 37-7 cells		
		Liver	Spleen	Kidney	Lung	Chest	Diaphragm
WT	Gross	5.1 ± 0.8	5.6 ± 1.3	0.2 ± 0.1	3.8 ± 0.4	0.4 ± 0.2	0.4 ± 0.3
	Microscopic	12 ± 1.5[b,c]	6.7 ± 0.6	0.0 ± 0.0[b,c]	25 ± 1.9[d]	ND	ND
NK2	Gross	>100	11 ± 1.5	1.0 ± 0.3	4.3 ± 0.4	0.6 ± 0.5	0.1 ± 0.1
	Microscopic	107 ± 8.1[b]	9.7 ± 0.8	0.8 ± 0.2[b]	29 ± 2.8[d]	ND	ND
HGF/SF	Gross	>50	21 ± 5.6	29 ± 12.5	3.0 ± 0.8	1.8 ± 1.3	0.8 ± 0.7
	Microscopic	39 ± 10[c]	ND	11 ± 3.9[c]	54 ± 14[d]	ND	ND

[a] Shown are the mean numbers of metastatic tumors per organ (± standard errors of the means) observed either grossly or microscopically in H&E-stained sections at their most prevalent sites following tail vein injections of either 10^5 (37-32) or 10^6 (37-7) cultured cells. All host mice (wild type [WT], NK2, and HGF/SF transgenics) were on the FVB/N genetic background, were between 2.5 and 3 months of age, and were sacrificed either at 21 days (37-32) or at 18 days (37-7) following intravenous injection. The cell line 37-32 robustly expressed c-Met (High-Met), while 37-7 expressed c-Met poorly (Low-Met); see the work of Otsuka et al. (35) for details on cell lines. ND, not done; in the case of the HGF/SF spleens, the 37-32 tumors had begun to fuse, preventing accurate quantification. For wild-type hosts, 59 and 22 mice were used for injection of 37-32 and 37-7 cells, respectively; for NK2 hosts, 29 and 14 mice were used for 37-32 and 37-7 cells, respectively; for HGF/SF hosts, 7 and 6 mice were used for 37-32 and 37-7 cells, respectively.

[b] $P < 0.0005$ comparing individual wild-type and transgenic organs.

[c] $P < 0.05$ comparing individual wild-type and transgenic organs.

[d] Not statistically different.

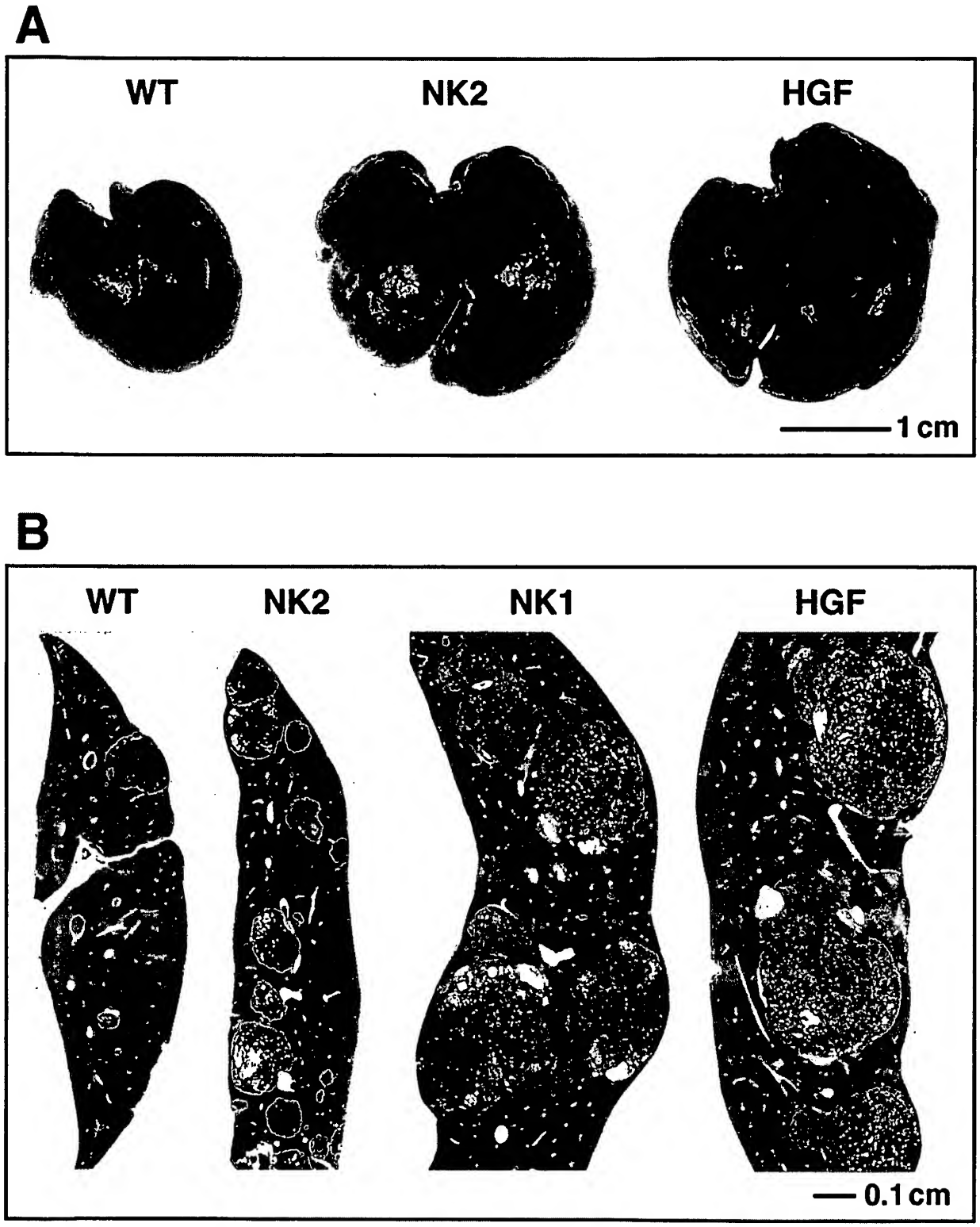

FIG. 7. NK2 enhances metastatic efficiency, but not growth, of high-Met-expressing melanoma cells. The figure shows results of analysis of liver metastasis of melanoma cells in genetically modified host mice. One million 37-32 melanoma cells were injected intravenously into the tail vein of wild-type (WT), NK2 transgenic, NK1 transgenic, and HGF/SF transgenic mice. (A and B) After 3 weeks, livers were examined grossly (A) and histopathologically (B) for the presence of metastatic tumors. Melanomas were immunohistochemically visualized (brown staining) using an anti-mouse TRP1 antibody. (C) Liver preparations from the genetically modified host mice were used to quantify both mean numbers of 37-32 melanoma cell metastases (white bars) and mean tumor sizes (black bars). Error bars indicate standard errors of the means. There was no statistically significant difference in the numbers of metastases per liver in the three transgenic lines. For mean tumor size, P value was <0.001 for NK2 versus either NK1 or HGF/SF; P value was 0.2 for NK1 versus HGF/SF.

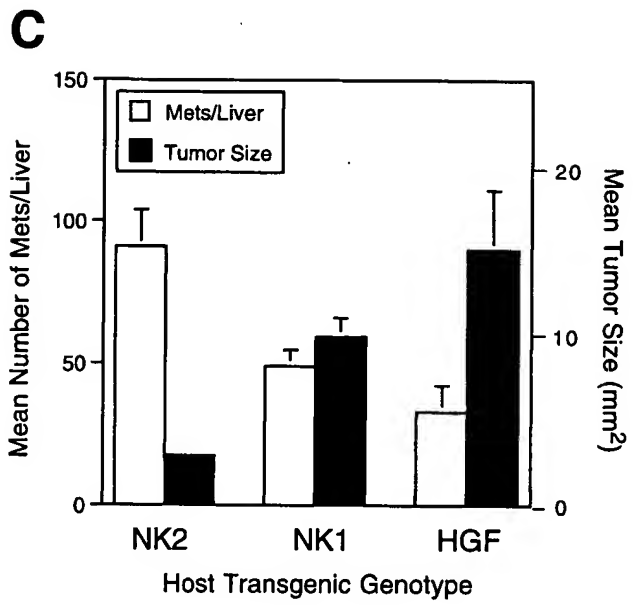

FIG. 7—*Continued.*

HGF/SF isoforms appear to engage and activate Met (18, 23, 31, 51, 60), we demonstrate here that Met autophosphorylation and MAPK activation are quantitatively different in 37-32 melanoma cells treated with NK2 than in those treated with HGF/SF. NK2 activities could also be explained by qualitative differences in Met tyrosine phosphorylation, which could in turn affect transducer recruitment and/or modify substrate target selection. Alternatively, differential signal persistence and/or intensity in individual pathways may account for the behavior of NK2. For example, the Met pathway(s) engaged in the induction of metastasis-associated behavior in vivo might require a weaker signal relative to other activities and still be triggered by NK2 despite its reduced ability to activate Met. Our in vitro data suggest that MAPK does not regulate this pathway. However, a credible candidate is the pathway(s) mediated by phosphotidylinositol 3-kinase, which can be activated by NK2 in some cultured cell lines (11) and in association with p110 can bind directly to the C-terminal Y^{1349}/Y^{1356} multifunctional docking site of Met (38, 39) and mediate cellular activities capable of contributing to metastatic spread (1, 16, 26, 27, 49, 57).

An important question then arises concerning the identity of the specific cellular activity, or activities, induced by NK2 that so efficiently facilitate metastasis in vivo. A number of studies on NK2 behavior in vitro proffer some insight. As originally described, NK2 was unable to induce, or could actually block, mitogenesis of cultured cells (6, 18, 30, 34). More recent in vitro studies showed that NK2 alone was incapable of inducing many other activities associated with metastasis, including in vitro invasion or urokinase-type plasminogen activator proteolysis (23), branching morphogenesis (23, 31), or angiogenesis (58). In contrast, NK2 was able to scatter canine MDCK cells in vitro (18) and stimulate cellular migration in a modified Boyden chamber assay system (60). Relying on these in vitro studies as a backdrop, our results present a strong case for enhanced cellular motility being critically influential in promoting metastasis in a bona fide animal model. However, enhanced NK2-induced scattering is almost certainly not sufficient for the acquisition of a full metastatic phenotype (16). In our melanoma model system, other requisite Met-mediated activities are likely being provided via the autocrine stimulation from the transgenic HGF/SF produced by the 37-32 cells themselves. Indeed, the fact that the subcutaneous growth of 37-32 tumor transplants is not effectively inhibited by host-generated NK2 suggests that the autocrine HGF/SF-Met signaling pathway is resistant to the antimitogenic effects of exogenous NK2. Such a conclusion raises serious doubts about the therapeutic efficacy of RTK antagonists whose malignant targets arise as a consequence of the creation of such autocrine RTK signaling loops.

Conclusions from the above discussion depend on the assumption that the metastatic enhancement induced by either NK2, NK1, or HGF/SF is a direct consequence of ligand binding to Met robustly expressed by the 37-32 malignant melanoma cells. However, an intriguing alternative possibility is that genetically modified host animals ectopically expressing these ligands become broadly permissive for metastasis, irrespective of the ability of the transplanted tumor cell to respond directly to Met ligands. HGF/SF and its variants could, for example, interact with host cells to induce subtle alterations in extracellular matrix or angiogenic networking, making these animals more susceptible to metastatic colonization in general. Although we cannot rule out this possibility at this time, the fact that low-Met 37-7 cells fail to demonstrate significantly enhanced metastatic behavior in these same transgenic hosts argues against it. This important issue will continue to be investigated; either way, these studies demonstrate the great experimental opportunity offered by an approach exploiting genetically modified host mice as tumor transplant recipients.

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